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Genetic Variability by *in Vitro* Mutagenesis and Arbuscular Mycorrhizal Fungal Symbiosis among Micropropagated Plants of *Agave vera-cruz* Miller

Dafedar H. Tejavathi[1*] • Kaivaram Gayathramma[2] • Rajashekharaiah Nijagunaiah[1]

[1] Department of Botany, Bangalore University, Bangalore-560 056, India
[2] Department of Biotechnology, Presidency College, Bangalore-560 024, India

Corresponding author: * **tejavathi_hanu@yahoo.com**

ABSTRACT

Agave vera-cruz Mill. is naturally found in semi-arid regions of India and provides a high quality fiber as a good source of income to farmers. Samples for the present study were obtained through direct and indirect organogenesis, arbuscular mycorrhizal fungi (AMF)-treated tissue-cultured plants as well as from tissue-cultured plants derived from exposure to gamma irradiation at 15 Gy for up to 20 min at 2-min intervals (physical mutagen) and ethyl methane sulfonate at 0.1 to 1% for 1 to 5 h (chemical mutagen). These, together with untreated plants were analyzed by amplified fragment length polymorphism (AFLP) markers. A phenotypically distinct variant derived from indirect organogenesis showed more polymorphism followed by mutagen-treated samples. Jaccard's coefficient of similarity was analyzed by the unweighted paired group method with arithmetic average (UPGMA) method. Plants obtained from mutagen-treated and untreated cultures formed two main clusters. Plants obtained through direct organogenesis and subsequently treated with AMF formed a separate sub-cluster indicating an altered molecular marker pattern. It is feasible to use *in vitro* culture methods coupled with mutagenesis and AMF symbiosis as a potential tool for increasing genetic variability of vegetatively propagated plants.

Keywords: AFLP, AMF, cluster analysis, *in vitro* mutation, somaclones
Abbreviations: BAP, 6-benzylaminopurine; **CTAB**, cetyl trimethylammonium bromide; **IBA**, indole-3-butyric acid; **MMS**, modified Murashige and Skoog medium; **NAA**, α-naphthaleneacetic acid; **Zea**, zeatin

INTRODUCTION

Agave vera-cruz Mill. (Agavaceae) is commonly known as grey aloe and is a perennial stout plant grown mainly in semiarid regions of India. It is a monocarpic taxon and flowers only once in its long life span of 25 to 30 years. It is mainly propagated vegetatively through suckers and bulbils. Though seeds are produced, viability is negligible (Escobar-Guzmán *et al.* 2008). Hence, conventional breeding programs cannot be employed to bring about any genetic improvement in these plants. Further, occurrence of a high degree of heterozygosity associated with polyploidy contributes to a complex inheritance and makes genetic analysis a difficult task in agaves (Gil-Vega *et al*. 2006). The genetic variability is usually negligible in vegetatively propagated plants where the progenies are clones with a narrow genetic diversity (Torres-Morán *et al.* 2010). However, the development of molecular markers and their application in analyzing genetic variations have demonstrated the existence of asexual genetic variability in both natural populations and micropropagated plants of agaves (Simpson *et al*. 2010). The genetic variability has been observed among the offsets obtained from *Agave fourcroydes* by Infante *et al*. (2003) and González *et al*. (2003) and recorded the differences at the molecular level using amplified fragment length polymorphism (AFLP) markers in the micropropagated plants (González *et al*. 2003) and also among the natural populations (Infante *et al*. 2003). *In vitro* propagated plants of *Agave tequilana* were found to be distinct from those propagated in field as analyzed by inverse ISTR molecular marker (Torres-Moran 2010). Genetic variations between the mother plant and their rhizomes in wild populations of *Agave angustifolia* were reported by Sánchez-Teyler *et al.* (2009) while analyzing the genetic variability

Fig. 1 (**A**) Source plants; (**B**). Leaf with spinocent margin, (**C**). Leaf with smooth margin.

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within and between geographically close populations by AFLP markers. After the publication of Larkin and Scowcroft (1981), there has been an upsurge of interest to understand the contributing factors for somaclonal variations arising from *in vitro* cultures.

Somaclonal variations may result from both the pre-existing genetic variation within the explants and variation induced during the callus phase of *in vitro* culture (Evans *et al*. 1984). However, the causes for such variations are not well understood though several explanations are offered including chromosomal and punctual mutations, somatic recombination, sister chromatid exchange, somatic genetic rearrangement, transposable elements and DNA methylation (Philips *et al*. 1994). DNA marker analysis provides an efficient method for screening tissue culture induced mutations and variations since the markers are not affected by environmental factors.. AFLP analysis of DNA markers has many advantages over other methods available for screening the genetic polymorphism at genomic level. AFLP markers showed differences at the molecular level between the offsets and bulbils produced asexually from the same mother plant of *A. tequilana* (Abraham-Juarz *et al.* 2009). AFLP is characterized by high reproducibility and does not require prior knowledge of sequences and has been widely used in the analysis of somaclonal variants (Gagliardi *et al*. 2007).

Mutation induction in combination with tissue culture and molecular analysis methods can be used as powerful technology to improve clonally propagated plants (Ahloowalia and Maluszynski 2001). Chemical and physical mutagens have been used for widening the genetic base of many vegetatively propagated plants. Since spontaneous mutations occur in extremely low frequency, *in vitro* mutation induction techniques are employed for the rapid creation and increase in variability. Many examples related to different vegetatively propagated species demonstrated that the combination of *in vitro* culture and mutagenesis is relatively inexpensive, simple and efficient (Patade and Suprasanna 2008). Hence, application of *in vitro* culture coupled with induced mutations can bring about desired productive traits and help in the selection of superior germplasm.

Arbuscular mycorrhizal fungi (AMF) form a symbiotic or mutual association with the roots of about 90% of the plants including angiosperms, gymnosperms, pteridophytes and bryophytes (Williams *et al*. 1994). AMF also play a significant role in producing healthy plantlets (Gianinazzi *et al*. 1988) and shortening of the acclimatization period of micropropagated plants (Salamanca *et al*. 1992). Since mycorrhizal inoculation improves nutritional qualities of the host, AM symbiosis is a potential factor to be considered to achieve nutritional security in the context of severity of deficiencies in arid and semi-arid regions (Subramanian *et al*. 2008). The microbial technology of mycorrhization of tissue cultured plants would offer an opportunity to apply molecular approaches to understand host-symbiotic interactions (Rai 2001). The aforesaid facts prompted us to study the genetic variation induced either by *in vitro* culture alone or in combination with *in vitro* mutagenesis and to evaluate the AMF treated normal and micropropagated plants of *Agave vera-cruz* by analyzing AFLP markers.

MATERIALS AND METHODS

Plant material

Standard *in vitro* culture techniques were followed to raise the tissue culture plants from untreated and mutagen treated shoot tip cultures (Tejavathi and Gayathramma 2005). The sample plants were selected randomly from the source plants maintained in the Botanical Garden of the Department of Botany, Bangalore University, Bangalore (**Fig. 1A**). Fourth leaf from the tip of 12-month-old plants of all the samples was used for the extraction of genomic DNA.

The source plants of samples

Sample A (normal plants): plants were raised from the 2-3-month-old saplings procured from the plantations in the semi-arid region of Ananthpur, Andhra Pradesh, and maintained in the Department Garden.

Sample B (tissue cultured plants through direct organogenesis): Regenerated shoots were obtained directly from shoot tip cultures on modified Murashige and Skoog medium (MMS) + α-naphthaleneacetic acid (NAA) (5.37 μM) + Zeatin (0.92 μM) + cholesterol (1034 μM). They were made to root on ½ MMS + indole-3-butyric acid (IBA) (9.84 μM) and the 2-month-old plantlets thus obtained are transferred to pots (28 × 12 × 24 : upper diameter × basal diameter × height in cm) containing 5 Kg of soil, sand and manure in a 1: 1: 1 ratio. All the chemicals and reagents were procured from HiMedia® (Mumbai, India, for plant tissue culture) (Tejavathi and Gayathramma 2005). Agaves contain the active principle, Hecogenin, a saponin. Cholesterol is one of the products produced in the biosynthesis of Hecogenin. Hence, cholesterol was used in the medium as a precursor for the biosynthesis of Hecogenin.

Sample C (tissue cultured plants through indirect organogenesis): Shoots were differentiated from the shoot tip derived callus on MMS + NAA (10.74 μM) + BAP (8.86 μM) and were made to root on ½ MMS + IBA (9.84 μM) and maintained as in sample B.

Sample D (tissue-cultured plants with smooth margin leaves): A few plantlets obtained through indirect organogenesis were phenotypically different with yellowish green leaves and smooth margin instead of bluish-green leaves with spinocent margin (**Figs. 1B, 1C**).

Sample E (normal plants treated with *Glomus mosseae*): Normal plants as in sample A were treated with *G. mosseae* at the age of 3 months. The inoculums containing about 200-250 spores/g were added at the rate of 25 g/pot.

Sample F (normal plants treated with *Glomus fasciculatum*): The procedure followed was same as sample E using *G. fasciculatum* as inoculum.

Sample G (tissue cultured plants treated with *G. mosseae*): Tissue cultured plants obtained through direct organogenesis as the sample B and were treated with *G. mosseae* at the age of 3 month as in the sample E.

Sample H (tissue cultured plants treated with *G. fasciculatum*): The procedure followed was same as sample E using *G. fasciculatum* as inoculum.

Sample I (tissue cultured plants obtained from physical mutagen treated shoot tip cultures through indirect organogenesis): The shoot tips were inoculated on MMS + NAA (10.74 μM) + BAP (8.86 μM) and irradiated with γ irradiation at the dosage of 15 Gy for 2 min intervals up to 20 min. and allowed to differentiate. Tissue cultured plant derived from the cultures exposed for 4 min of irradiation was selected since it was found to be the optimum with large number of multiple shoot induction through indirect organogenesis.

Sample J (tissue cultured plants obtained from chemical mutagen-treated shoot tip culture through indirect organogenesis): The shoot tips were incubated in ethyl methane sulfonate (EMS) at concentrations ranging from 0.1 to 1% for 1 to 5 h. After the treatment they were inoculated onto MMS + NAA (10.74 μM) + 6-benzylaminopurine (BAP) (8.86 μM) for indirect organogenesis and thus obtained shoots were made to root as other samples. The regenerated plant obtained from the culture treated with 0.2% EMS for 2 h was selected since it was found to be the optimum treatment with large number of shoots.

DNA extraction

A modified CTAB method proposed by Keb-Llanes *et al*. (2002) particularly for Agavaceae was followed to extract the genomic DNA from 300 mg of tissue. The protocol involved precipitation and resuspension of DNA for 3 times at the end of the preparation, which increased the DNA digestibility, and the sharpness of AFLP bands. Hence, a homogenous DNA unshared with RNA contamination was obtained. The purity, concentration and integrity of DNA were monitored by electrophoresis on 0.8% agarose gel in TBE Buffer (Sambrook *et al*. 1989) and λ DNA. All the reagents

Table 1 Summary of AFLP fragments in ten samples of *Agave vera-cruz*.

Samples	AFLP markers			Polymorphic bands			Percent polymorphism			TFM	TPB	TPP
	I	II	III	I	II	III	I	II	III			
A	53	43	47	10	12	11	18.8	25.5	23.4	143	33	23.0
B	52	44	45	13	14	13	25.0	31.8	28.8	141	40	28.3
C	58	45	42	15	12	14	25.0	26.6	33.3	145	41	28.6
D	58	62	46	21	18	17	36.0	39.1	36.9	166	56	35.8
E	61	49	41	11	13	11	18.8	26.5	26.8	151	35	23.1
F	52	50	44	13	10	12	25.0	20.0	27.2	146	35	23.9
G	59	46	44	13	13	11	22.0	28.0	25.0	149	37	24.8
H	52	50	46	14	14	12	26.0	24.0	26.0	148	40	27.0
I	54	50	45	19	13	15	35.1	26.0	33.3	149	47	31.5
J	51	48	45	20	12	14	39.2	29.1	31.3	144	46	31.9
Total	550	487	445	149	131	130	27.0	26.8	29.2	1482	410	27.6

TFM - total number of AFLP markers; TPB - total number of polymorphic bands; TPP - total percent polymorphism
I: E-ACG/M-CTC
II: E-ACG/M-CTG
III: E-ACG/M-CTT

and primers used in AFLP were from Bangalore Genei®, India.

AFLP analysis

AFLP fragments were generated based on protocol of Vos *et al*. (1995) with minor modifications. It was ensured that the DNA samples were intact and PCR conditions were uniform in both pre-amplification and selective amplification conditions. The protocol for AFLP analysis was optimized for concentration of template DNA, Primers, Taq polymerase, dNTP mix and PCR buffer. It was found that 5 µl of template DNA, 1.5 U of 0.5 µl of *Taq* polymerase, 2 mM of dNTP mix, 2 µl of 10X PCR buffer and 2 µl of AFLP primers as optimal. DNA amplification was performed with 15 sets of primer combinations comprising *Eco*RI-ACG/*Eco*RI-AGG/*Eco*RI-ACC with six different primers of *Mse*I. Of these primer combinations, *Eco*RI-ACG with three different primers of *Mse*I showed reproducible and well resolved bands. These primer combinations were selected finally based on their reproducibility (**Table 1**): E-ACG/M-CTC, E-ACG/M-CTG, E-ACG/M-CTT.

Pre- and selective amplification

5 pM of *Eco*RI and 50 pM of *Mse*I adapters were ligated using 0.5U of T4 DNA ligase and confirmed by running agarose gel electrophoresis. Ligated DNA was diluted two folds with sterile distilled water and used as a template for PCR pre-amplification. The PCR (Corbet and MWJ Research hot spot thermocycler) profile was programmed as follows: 94°C for 30 s for denaturation, 56°C for 60 s for DNA extension, 72°C for 60 s for annealing. For selective amplification, different primer combinations of *Eco*RI and *Mse*I each having 2-3 selective nucleotides at the 3′ end were used. The PCR profile for selective amplification as follows: 94°C for 30 s, 65°C for 30 s reduced by 7°C in each sequence cycle to 58 and 72°C for 60 s. This step was run for 11 cycles for denaturation. The second step was run for 24 cycles for extension as follows: 94°C for 60 s, 56°C for 30 s and finally 72°C for 60 s. Then the electrophoreses of amplified products were done on 4.5% polyacrylamide gel under denatured conditions and visualized with silver staining. The number of bands resolved per amplification was primer dependent and varied from one sample to the other.

Data analysis

The AFLP profile of the samples were scored as 1 for band present or 0 for absent in a binary matrix. The genetic similarity was estimated according to Jaccard's coefficient and clusters were generated using unweighted pair group method with arithmetic average (UPGMA). All the computations were carried out using NTSYS-Pc ver. 2 (Rohlf 1998). Percent polymorphism was calculated as follows:

$$\%\text{Polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

RESULTS

A total of 1482 fragments were detected in AFLP profile of 10 samples in the three primer combinations (**Fig. 2**). Among these, about 27.6% of polymorphism and 72.4% of monomorphism was recorded. Average fragment length was found to be 120 bp, but fragments shorter than 100 bp long were also detected and were monomorphic. Among all the three primer combinations, the maximum bands of 550 were observed in E-ACG/M-CTC, whereas minimum of 445 bands in E-ACG/M-CTT primer combinations. The maximum polymorphism (29.2%) was observed in E-ACG/M-CTT and minimum polymorphism (26.8%) in E-ACG/M-CTG primer combinations (**Table 1**).

The AFLP profile of sample D, which is a phenotypic variant showed maximum number of polymorphic bands with 35.8%. Least number of polymorphic bands (23.0%) was found in sample A, which represents normal plants that is comparable to the samples E (23.1%) and F (23.9%) which are again normal plants but treated with *G. mosseae* and *G. fasciculatum*, respectively. The sample I and J were derived from mutagen treated cultures also showed more polymorphic bands (31.5% and 31.9%) but less than the sample D (35.8%).

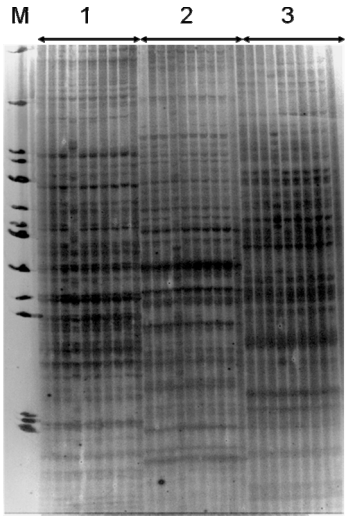

Fig. 2 AFLP profile of 10 samples. Lane: 1, E-ACG/M-CTC, 2. E-ACG/M-CTG, 3. E-ACG/M-CTT.

Similarity co-efficient and dendrogram analysis

Two main clusters were formed when AFLP data was computed using NTSYS-Pc and UPGMA analysis (**Fig. 3**). Cluster 1 has all the samples derived from direct and indirect organogenesis along with the normal and tissue cultured AM-treated plants. Cluster 1 has two groups 'a' and 'b'. Group 'a' includes normal and tissue cultured plants

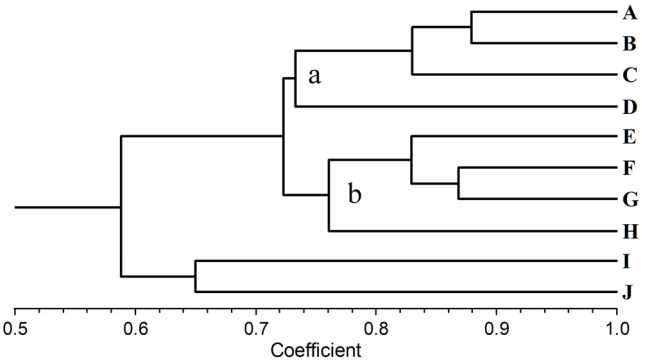

Fig. 3 Dendrogram of AFLP profile of 10 samples (A to J) generated using Jaccard's similarity index and UPGMA cluster analysis.

(samples A to D), while group 'b' constitutes AMF normal and tissue cultured plants (samples E to H). Whereas cluster 2 comprises the samples that were raised through *in vitro* mutagenesis from shoot tip callus (samples I and J). In the main cluster 1, sample D is phenotypically different from other plants, which was derived from callus cultures showed maximum polymorphism (35.8%) and similarity co-efficient of 0.72 with the normal plants whereas samples I and J, obtained through *in vitro* mutagenesis, share least similarity co-efficient with the normal plants.

DISCUSSION

Since *Agave vera-cruz* exhibits limited sexual reproduction the availability of genetic variability through sexual reproduction is negligible (Robert *et al.* 2006). In such conditions induced mutations play an important role for the genetic improvement. As suggested by Novak (1991), induced mutations in combination with *in vitro* culture techniques form an effective tool for plant improvement. The 10 samples used for genetic analysis in the present investigation are mainly grouped into two main clusters. Cluster 1 represented the tissue culture progenies along with normal plants whereas cluster 2 included tissue culture progenies derived from mutagen treated cultures. Further, cluster 1 has two distinct groups, designated as 'a' and 'b'. Group 'a' constituted normal plants (sample A), plants derived from direct organogenesis (sample B) and plants from indirect organogenesis (samples C and D). Whereas group 'b' included AMF-treated plants.

Sample A and B share more similarity of about 0.88% confirming that the direct organogenesis does not usually result in any tissue culture-induced variations and the resulting progenies are genetically more stable. In contrast, samples C and D were obtained from callus cultures, though related in their origin, they diverge and share different similarity value as Larkin and Scrowcroft (1981) pointed out that organogenesis from callus cultures results in the occurrence of somaclonal variations. Genetic variations are generally more pronounced in the plants regenerated through callus phase rather than the direct regeneration. This has manifested in the sample D, which is phenotypically distinct from other samples in having yellowish-green colored leaves with smooth margin instead of bluish-green, leaves with spinocent margins. AFLP profile of this sample obtained from all the three primer combinations also indicates its uniqueness in having more polymorphic bands and requires further characterization. However, in *A. fourcroyds* the micropropagated plants through somatic embryogenesis from callus culture showed less variation from their mother plants (Gonzalez *et al*. 2003). Polanco and Ruiz (2002) also reported about 99.4% of shared AFLP fragments among the regenerated plants of *Arabidopsis thaliana* from the root callus cultures while in *Musa* spp. (Newbury *et al*. 2000) and *Coffea arabica* (Sánchez-Teyer *et al*. 2003) using RAPD and AFLP respectively, it was found that *in vitro* derived plants are different from source plants. Sample C though genetically different in having 41 polymorphic bands, morphologically resembles the samples A and B, however with minor variations like length and breadth of the leaves. Culture conditions and nature of the explants can induce genetic and epigenetic variations.

Cultured plant cells may undergo somaclonal variation which is manifested in the alteration of either phenotypic or genotypic traits. The tissue culture environment may also cause a general disruption of cellular controls leading to a numerous genomic changes in the regenerants. Previous studies indicate that changes in DNA methylation can occur when plants are exposed to *in vitro* conditions (Phillips *et al*. 1994). Repeat-induced point mutations occur frequently in the newly methylated sequences as a result of deamination of methylated cytosines. Phillips *et al*. (1994) are of the opinion that better understanding of these two mechanisms methylation and repeat-induced point mutation will provide an insight into the factors that are responsible for *in vitro* culture induced variations.

The four samples E, F, G and H which are grouped in group 'b' are AMF-treated plants. Samples E and F are normal plants treated with *G. mosseae* and *G. fasciculatum*, respectively whereas samples G and H are tissue cultured plants derived through direct organogenesis and treated with *G. mosseae* and *G. fasciculatum*, respectively. The symbiotic association of mycorrhizal fungi with roots of higher plants helps in the acquisition of immobile elements such as phosphorus, sulphur and zinc (Russo *et al*. 1993) and to overcome the stressed situation and reduce mortality of micropropagated plants (Subhan *et al*. 1998). Since the mycorrhizal association helps the host in uptake of essential elements and water, the increase in biomass is eminent. High polyploidization and decondensed chromatin were observed in AMF-treated plants which are usually associated with high metabolic activity (Berta *et al*. 2000). Differential gene expression patterns were observed in AMF-treated and untreated tomato plants. However, the mechanism of how the symbiotic association is responsible for inducing genetic variation is not clearly understood and needs further investigation.

Cluster 2 includes samples I and J which are tissue cultured plants raised from γ irradiated and EMS treated callus cultures respectively stand out from all other tissue cultured plants. Induced mutations have contributed significantly for plant improvement and more than 2500 mutant varieties have been developed through mutation breeding (Patade and Suprasanna 2008). The success of *in vitro* mutation is due to the occurrence of dominant mutations (Larkin and Scowcroft 1981) and regeneration of large number of plantlets within a short span of time from mutagen treated cultures (Tejavahi *et al*. 2010). Patade and Suprasanna (2008) were able to isolate agronomically desirable mutant lines in sugarcane through *in vitro* mutagenesis. Mutagens have been utilized to widen the genetic base for breeding of many vegetatively propagated plants.

Agave tequilana var. 'Azul', one of the commercially important plants of Mexico, because of overexploitation and limited germplasm base owing to Federal law has become unalterable to both pest attack and adverse environmental conditions. When analyzed by RAPD markers by Gil-Vega *et al*. (2001), it was found that only 1 of 124 RAPD products was polymorphic with 0.8% polymorphism suggesting a very narrow genetic base for variation. However, through AFLP analysis Gil-Vega *et al*. (2006) could notice 78 of 301 polymorphic bands (26%). They attributed these differences in these two studies to different techniques involved and concluded that the gene pool of *A. tequilana* var. 'Azul' is not as narrow as once thought and can be exploited by employing newer breeding programmes including *in vitro* culture to improve the production of high yielding genotypes. Induced mutations coupled with *in vitro* culture can be used as a powerful tool in this type of species where there is limited stock but yet are commercially viable germplasm.

The mechanism for the occurrence of the asexual genetic variations is not clearly understood. Infante *et al*. (2003)

suggested that the observed genetic variations in agaves might be due to the accumulation of somatic mutations. Their conclusion was based on the mathematical models proposed by Otto and Orive (1995) and Pineda-Krch and Fagestrom (1999) who derived the somatic cell mutations from the fixation of mutation in any one of the somatic cells in the apical meristem. Further, Infante *et al*. (2003) believed that high degree of polyploidy in agaves could result in the formation of varying number of chromosomes. *Agave vera-cruz* is a polyploid species with 2n = 60 (x = 5) chromosomes. Since tissue cultured plants in the present study were derived from shoot tip cultures, the mechanisms proposed by Infante *et al*. (2003, 2006) could be the source of genetic variations observed among the tissue cultured plants in combination with *in vitro* mutagenesis and host-symbiont interactions. Mutation breeding, molecular marking of the selected mutants and multiplying them by *in vitro* techniques have tremendous potential in genetic improvement of vegetatively propagated plants. Genetic analysis of AM fungal treated and untreated micropropagated plants through molecular markers can give an insight into the subtle interaction operating during symbiosis.

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