Assessment of Genetic Stability on *In Vitro* And *Ex Vitro* Plants of *Ficus Carica* Var. Black Jack Using Issr and Damd Markers

Ankita Rajendra Parab  
Universiti Sains Malaysia

Chew Bee Lynn  
Universiti Sains Malaysia

Sreeramanan Subramaniam  
Universiti Sains Malaysia

Research Article

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Abstract

In vitro propagation has been significant in producing a large number of genetically stable regenerated plants. Regenerated Ficus carica var. Black Jack plantlets were established using woody plant medium (WPM) supplemented with 20 µM 6-Benzylaminopurine (BAP) and 8 µM indole-3-acetic acid (IAA) under different light treatments such as normal fluorescent white light (60 µmol.m⁻².s⁻¹), and four different LED spectra, white (400–700nm), blue (440nm), red (660nm) and blue + red (440nm + 660nm). Genetic stability analysis was performed on the in vitro and ex vitro plants of Ficus carica var. Black Jack. Ten (10) primers of each ISSR and DAMD molecular marker were used to assess the genetic stability of the eight (8) samples of Ficus carica var. Black Jack, acquired over two years. The findings of this study revealed that inter simple sequence repeats (ISSR) and directed amplification of minisatellite DNA (DAMD) markers (DNA primers) are efficient in determining the polymorphism and monomorphism percentage among the in vitro and ex vitro samples of Ficus carica var. Black Jack. ISSR markers showed 97.87% of monomorphism whereas DAMD markers showed 100% monomorphism. Polymorphism of 2.13% was observed for the UBC840 ISSR – DNA primer which was negated under the genetic similarity index analysis for the eight samples. It is recommended that genetic stability analysis should be performed for long-term maintenance of micropropagated plants.

1. Introduction

Micropropagation is a technique widely used for the commercialization of many woody plant species. Producing a whole plant using an explant is termed micropropagation. Varieties of plants will produce different responses under different growth conditions. In vitro regeneration of plants involves the use of various plant growth regulators (PGRs) (Shen et al. 2010) and growth conditions such as the number of subcultures (KiahYann et al. 2012) and the light intensities (Cioč et al. 2018) to achieve optimum growing conditions for plants (Yadav et al. 2013; Prameela et al. 2015; Rohela et al. 2019).

Micropropagation in plants solely depends on the phenomenon of totipotency among plants. The application of micropropagation includes growing any plant species on a large scale commercially, clonal propagation of endangered species of plants, somaclonal variations, producing transgenic plant cell lines and sterile hybrids with enhanced properties, and acquiring essential secondary metabolites. The micropropagation system has a huge advantage in the commercial market of plant tissue culture, owing to its excellent multiplication techniques and viability. Using highly dividing meristematic tissues of the apical buds, leaves, or roots are used as explants to achieve plant regeneration through various techniques such as organogenesis or somatic embryogenesis. (Rathore et al. 2005; Iliiev et al. 2010; Ružičet al. 2012).

The technique of regeneration of plants generally includes steps like acquiring the explant from a suitable mother plant, using PGRs in the culture media, and culturing in vitro plants on a suitable media (Asadi et al. 2009). Many factors affect the in vitro growing plant cultures. Using different PGRs is a major contributing factor to the change in plant genetics. The growth conditions such as the use of different light spectra or even the number of times the culture is subculture also affect the plant genetics (Huang et al. 2009; Butiuć-Keul et al. 2016; Baghel and Bansal, 2017). Somaclonal variations in plants can also occur due to the difference in the in vitro growth conditions such as different light spectra and the photoperiod. Hence, evaluation of the genetic stability of the in vitro regenerated plants is a very crucial step that helps in concluding a successful micropropagation technique. The regeneration capabilities of the totipotent plants from a single cell into a whole new plant may introduce genetic changes once the tissues are dissected and obligated to a pathway of cell division using specific growth regulators (Seiler et al. 2017; Ngilah et al. 2018).

The alterations caused on the plants due to the somaclonal variations can be in the form of change in plant morphology, variation in chromosome number, expression of genes, protein profile, and DNA sequences. Overall the assessment of the genetic stability among the regenerated plantlets is based on various analyses such as biochemical, cytological, and molecular analyses (Leva et al. 2012; Garcia et al. 2019). The studies on morphological and genetic variations in forty – five (45) different varieties of figs (Ficus carica L.) were reported by Oukabli et al. (2003). They have concluded that regional constraint has an impact on the clonal variations among the different cultivars based on the environmental factors of the particular regions. Reports have also proven that overall genetic stabilities were maintained in the regenerated plants such as wasabi (Wasabia japonica) (Matsumoto et al. 2013), bananas (Musa acuminata) (Lamare and Rao, 2015), Henckelia incana (Prameela et al. 2015), melons (Melonia cultivars) (Parag et al. 2010), and Rauwola tetraphylla (Rohela et al. 2019).
Recently, polymerase chain reaction (PCR) based techniques have been known to be efficient for genetic studies among plants. PCR based single primer amplification reaction (SPAR) techniques using microsatellites, simple sequence repeats (SSR), inter simple sequence repeats (ISSR), random amplified polymorphic DNA (RAPD), and directed amplification of minisatellite DNA (DAMD) primers to analyse and detect the genetic stability among the in vitro generated plants are the most common practices in plant tissue culture experiments. Techniques which utilise single primers to amplify the DNA in PCR reactions are generally applicable universally (Zoghlami et al. 2012; Lamare and Rao, 2015; Baghel and Bansal, 2017; Rohela et al. 2019). The use of ISSR and DAMD markers has proven to be unbiased and cost-effective as compared with the RAPD markers (Singh et al. 2014; Purayil et al. 2018; Meena et al. 2019). Consequently, the present study uses ISSR and DAMD markers to evaluate the genetic stability of the regenerated multiple shoots, mother plant and ex vitro acclimatised plants of Ficus carica var. Black Jack.

Prior to the current study, a full-strength woody plant medium (WPM) (McCown and Lloyd, 1981) supplemented with 20 µM 6-Benzylaminopurine (BAP) was used to acquire multiple shoots on the axenic apical buds of Ficus carica var. Black Jack. The apical buds were grown under five different light treatments namely, normal fluorescent white light (60 µmol.m⁻².s⁻¹), and four different LED spectra, white (400nm – 700nm) [W], blue (440nm) [B], red (660nm) [R] and blue + red (440nm + 660nm) [BR]. The multiple shoots were rooted using WPM supplemented with 20 µM BAP and 8 µM indole-3-acetic acid (IAA). The rooted plantlets were used for ex vitro acclimatization using Biochar soil. The current study was intended to assess the genetic stability of the regenerated plantlets compared with the mother plant and ex vitro acclimatised plants of Ficus carica var. Black Jack using ISSR and DAMD markers.

2. Materials And Methods

2.1 Deoxyribonucleic acid (DNA) extraction from the mother plant, in vitro plants and ex vitro acclimatised plants of Ficus carica var. Black Jack

For the amplification and analysis of genomic DNA of Ficus carica var. Black Jack, eight types of treatments were selected: 2 year old mother plant [M], in vitro regenerated plants under normal fluorescent white light (60 µmol.m⁻².s⁻¹) (in vitro control) [C], 4 different LED spectra namely white (400–700nm) [W], blue (440nm) [B], red (660nm) [R] and blue + red (440nm + 660nm) [BR], and ex vitro acclimatized plants from normal white light [EX_N] and ex vitro acclimatized plants from blue + red (440nm + 660nm) [EX_BR] treatments. Samples collected from in vitro treated plants were five months old. The control is considered to be the mother plant [M] which is compared with 7 treatment, namely in vitro grown plants under normal fluorescent white light [C], in vitro plants grown under 4 different LED spectra; white [W], blue [B], red [R], blue + red [BR] and two samples from 8 months old ex vitro acclimatised plants [EX_N] and [EX_BR].

2.2 DNA extraction procedure

Genomic DNA isolation was performed using the ‘Genomic DNA Purification Kit’ (Promega Wizard® Genomic DNA Purification Kit, Madison, WI 53711 - 5399 USA). The quality of DNA was checked by electrophoresis on 1.5% agarose gel. The concentration (ng/µL) and purity (A260/A280) of the DNA extract was quantified using nanodrop spectrophotometer (ASP-2680, ACTGene Inc., New Jersey, USA).

2.3 Amplification of the genomic DNA using ISSR and DAMD markers

Genetic stability among the mother plant and regenerated plants (in vitro plants under normal incandescent light and six different LED spectra treatments) were analysed using ISSR and DAMD DNA markers. Chemicals and solutions used for the following study were purchased from the 1st Base Malaysia (exTEN 2X PCR Master Mix). The exTEN Master Mix contained 0.08 U/µl exTEN DNA Polymerase, 400µM dNTP Mix, 3mM MgCl₂, reaction buffer, and a PCR enhancer.

2.3.1 PCR amplification using ISSR – DNA method

The composition of molecular reagents in the polymerase chain reaction (PCR) tubes, is as per mentioned in Table 1. A total of ten ISSR primers (Devi et al. 2014) were screened for the following study to assess the genetic stability of Ficus carica var. Black Jack (Table 2). Overall, seven primers produced precise and reproducible bands. Thus, these seven primers were used in subsequent
performed in the volume of 25 µL in 200µL tube (Axygen Inc. California USA). The PCR profile was performed at an initial denaturation temperature of 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C (depending on the melting Temperature \([Tm – 5°C]\)) for 30 seconds, extension at 72°C for 1 minute and then a final extension at 72°C for 10 minutes.

| Components                      | Volume (µL) |
|---------------------------------|-------------|
| exTEN 2X PCR Master Mix         | 12.5        |
| Primer                          | 2.5         |
| Genomic DNA Template            | 3           |
| Sterile de-ionised water        | 7           |
| Total                            | 25          |

**Table 1**

Components for a 25 µL reaction volume for PCR process.

A total of ten DAMD primers (Devi et al. 2014) were screened for the following study to assess the genetic stability of *Ficus carica* var. Black Jack (Table 3). A total of, seven primers produced precise and reproducible bands. Thus, these primers were used in subsequent DAMD analysis. The DAMD primers were synthesised from 1st Base Malaysia DNA amplification. The PCR amplification was performed in the volume of 25 µL in a 200µL Eppendorf tube (Axygen Inc. California USA), and the composition of molecular reagents (Table 1). The PCR profile was performed at an initial denaturation temperature of 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C (depending on the melting temperature \([Tm – 5°C]\)) for 30 seconds, extension at 72°C for 1 minute and then a final extension at 72°C for 10 minutes.

| Primers | Sequence 5’ – 3’ | Melting Temperature (°C) | Annealing Temperature (°C) |
|---------|------------------|--------------------------|---------------------------|
| N3      | GAGAGAGAGAGAGAGAYT | 47.2                    | 47.4                      |
| N6      | CACCACCACGC       | 72.7                    | 44.7                      |
| N7      | GAGGAGGAGGC       | 72.7                    | 41.0                      |
| N8      | CACACACACACAGT    | 50.0                    | 44.7                      |
| N9      | ACACACACACACACACAG | 50.0                   | 52.9                      |
| UBC840  | GAGAGAGAGAGAGAGAYT | 47.0                    | 42.0                      |
| UBC811  | GAGAGAGAGAGAGAC   | 52.9                    | 46.8                      |
| UBC818  | CACACACACACACACAG | 51.0                    | 46.0                      |
| UBC 864 | ATGATGATGATGATGATG | 43.6                   | 38.6                      |
| UBC855  | ACACACACACACACACYT | 47.2                   | 53.1                      |

**Table 2**

List of ISSR – DNA Primers used for the genetic stability assessment of the samples of *Ficus carica* var. Black Jack.

### 2.3.2 PCR amplification using DAMD – DNA method

A total of ten DAMD primers (Devi et al. 2014) were screened for the following study to assess the genetic stability of *Ficus carica* var. Black Jack (Table 3). A total of, seven primers produced precise and reproducible bands. Thus, these primers were used in subsequent DAMD analysis. The DAMD primers were synthesised from 1st Base Malaysia DNA amplification. The PCR amplification was performed in the volume of 25 µL in a 200µL Eppendorf tube (Axygen Inc. California USA), and the composition of molecular reagents (Table 1). The PCR profile was performed at an initial denaturation temperature of 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C (depending on the melting temperature \([Tm – 5°C]\)) for 30 seconds, extension at 72°C for 1 minute and then a final extension at 72°C for 10 minutes.
Table 3
List of DAMD – DNA Primers for the genetic stability assessment of the samples of *Ficus carica* var. Black Jack.

| Primers  | Sequence 5’ – 3’ | Melting Temperature (˚C) | Annealing Temperature (˚C) |
|----------|------------------|--------------------------|----------------------------|
| 6_2H_t   | AGGAGGAGGGGAAGG  | 52.4                     | 47.4                       |
| FV11e8_C | CCTGTGTGTGTGCAT  | 49.1                     | 44.1                       |
| HVR      | CCTCTCTCCTCTCT   | 47.6                     | 42.6                       |
| HVA      | AGGATGGAAAGGAGGC | 51.0                     | 46.0                       |
| 14C2     | GGCAGGATTGAAGC   | 46.5                     | 41.5                       |
| 14C3     | GAGGTTGGCGGCTCT  | 57.9                     | 52.9                       |
| URP1F    | ATCCAAGTCCGAGACAACC | 56.8                  | 51.8                       |
| URP2F    | GTGTGGCAGTCTGTGGGA | 59.1                  | 54.1                       |
| URP25F   | GGACAAGAAGGATGGA  | 55.0                     | 50.0                       |
| UBC861   | ACCACCACCACCACCACC | 60.6                  | 55.6                       |

2.4 Agarose gel electrophoresis (AGE) analysis

PCR amplified products were separated using electrophoresis in 1.5% (w/v) agarose. Dissolve 0.6 gm agarose in 40mL 1×Tris-Borate-EDTA (TBE), melt it in a microwave oven, and leave till it cools down. Once it was cooled, 2µL red – safe stain (Intron Biotechnology™) was added. The gel was cast in a Mini Gel Caster (Bio-Rad Laboratories, Inc., USA) and was allowed to solidify at room temperature for 20 minutes before transferring to the Wide Mini Sub-Cell® GT agarose gel electrophoresis System (Bio-Rad Laboratories, Inc., USA) base filled with 1X TBE buffer. Subsequently, 3 µL of Thermo Scientific GeneRuler and 100bp plus DNA Ladder ready to use (Thermo Scientific, EU, Lithuania) were loaded into the well to function as the molecular markers. Ready to use PCR amplified products were then loaded into the following wells (5 µL) (Fig. 1). The electrophoresis system was attached to the PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Inc., USA). The samples were electrophoresed at 70V for 60 minutes.

2.5 Determination of polymorphism analysis

Genetic similarity index for the samples of *Ficus carica* var. Black Jack was assessed. The visible bands were manually scored as 1 or 0 for the presence or absence of the band. Similarity index was calculated according to Nei and Li (1979) and Harirah and Khalid (2006):

\[
\text{Similarity index (Si)} = \frac{2N_{xy}}{N_{x} + N_{y}}
\]

Where, \( N_{xy} \) = number of monomorphic bands between the control and treatment groups

\( N_{x} \) = total number of bands in the control group

\( N_{y} \) = total number of bands in the treatment group

The polymorphism percentage among the samples was calculated using the formula,

\[
\text{Polymorphism percentage(\%)} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100
\]

3. Results

3.1 ISSR – DNA analysis
For the ISSR analysis, seven out of the selected ten primers produced precise and reproducible band outcomes (Fig. 2; Table 4). In general, all 7 ISSR primers resulted in 24 bands from each of the treatments the mother plant sample [M], in vitro control [C], white LED [W], blue LED [B] and 23 bands each from the red LED [R], blue + red LED [BR], ex vitro acclimatised plants from normal white light [EXN] and ex vitro acclimatised plants from blue + red [EXBR] treatments. The sizes of the amplification products generated were ranged from 100 to 800 bp with scorable bands which were produced from each primer ranged from 1 (N8) to 7 (UBC864) bands (Table 4).

The genetic stability between the mother plant and the in vitro regenerated plants from seven different treatments was compared. Banding profiles obtained from the ISSR – DNA primers showed a similar banding pattern for all the treatments. Thus, the amplicons produced were monomorphic. Results indicate that there was 2.12 % polymorphism and 97.87 % monomorphism (Table 7). All primers, except one (UBC840), produced an SI of 1.0. Based on Table 5, the SI value of approximately 0.95 was calculated among the 8 samples of Ficus carica var. Black Jack for primer UBC840. Thus based on the SI partial polymorphism can be conferred in primer UBC840.

### Table 4

| Primers | Mother plant (M) | in vitro control (C) | W | B | R | BR | EXN | EXBR | No. of monomorphic Bands | No. of polymorphic Bands | SI Index | Length of amplified DNA fragments (bp) |
|---------|------------------|----------------------|---|---|---|----|-----|------|--------------------------|--------------------------|----------|--------------------------------------|
| N6      | 2                | 2                    | 2 | 2 | 2 | 2  | 2   | 2   | 0                        | 1.0                     | 200–600   |                                       |
| N7      | 2                | 2                    | 2 | 2 | 2 | 2  | 2   | 2   | 0                        | 1.0                     | 150–300   |                                       |
| N8      | 1                | 1                    | 1 | 1 | 1 | 1  | 1   | 1   | 0                        | 1.0                     | 200–300   |                                       |
| UBC840  | 6                | 6                    | 6 | 6 | 5 | 5  | 5   | 5   | 1                        | 0.95                    | 100–500   |                                       |
| UBC811  | 3                | 3                    | 3 | 3 | 3 | 3  | 3   | 3   | 0                        | 1.0                     | 200–600   |                                       |
| UBC818  | 3                | 3                    | 3 | 3 | 3 | 3  | 3   | 3   | 0                        | 1.0                     | 300–600   |                                       |
| UBC864  | 7                | 7                    | 7 | 7 | 7 | 7  | 7   | 7   | 0                        | 1.0                     | 200–800   |                                       |
| Total   | 24               | 24                   | 24| 24| 23| 23 | 23  | 23  | 1                        | -                        | -         | -                                    |
Table 5
Genetic similarity index between 8 samples of *Ficus carica* var. Black Jack using ISSR – DNA primer (UBC840) analysis.

| *Ficus carica* var. Black Jack | M     | C     | W     | B     | R     | BR    | EX_N  | EX_BR |
|--------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| M                             | 1.0000| 1.0000| 1.0000| 0.9090| 0.9090| 0.9090| 0.9090| 0.9090|
| C                             | 1.0000| 1.0000| 1.0000| 0.9090| 0.9090| 0.9090| 0.9090| 0.9090|
| W                             | 1.0000| 1.0000| 1.0000| 0.9090| 0.9090| 0.9090| 0.9090| 0.9090|
| B                             | 1.0000| 1.0000| 1.0000| 0.9090| 0.9090| 0.9090| 0.9090| 0.9090|
| R                             | 0.9090| 0.9090| 0.9090| 0.9090| 1.0000| 1.0000| 1.0000| 1.0000|
| BR                            | 0.9090| 0.9090| 0.9090| 0.9090| 1.0000| 1.0000| 1.0000| 1.0000|
| EX_N                          | 0.9090| 0.9090| 0.9090| 0.9090| 1.0000| 1.0000| 1.0000| 1.0000|
| EX_BR                         | 0.9090| 0.9090| 0.9090| 0.9090| 1.0000| 1.0000| 1.0000| 1.0000|
| Average                       |       |       |       |       |       |       |       | 0.9480|

\[ a \] SI value was calculated according to the arithmetic approach by Nei and Li (1979); Huijiang et al. (2005) and Harirah and Khalid (2006). Similarity index denotes the relationship between the amplified DNA fragments among the samples. This relationship coefficient should be \( 0 \leq F_{xy} \leq 1 \)

### 3.2 DAMD – DNA analysis

A total of ten DAMD primers were used to analyse the genomic DNA acquired from the samples of *Ficus carica* var. Black Jack. Seven (7) DAMD primers produced well-separated and reproducible band outcomes (Fig. 3; Table 6). Overall, 23 monomorphic bands were produced from samples collected from each treatment. Namely, mother plant sample [M], *in vitro* control [C], white LED [W], blue LED [B], red LED [R], blue + red LED [BR], *ex vitro* acclimated plants from normal white light [EX_N] and *ex vitro* acclimated plants from blue + red [EX_BR] treatments. The amplified products generated band sizes from 150 to 1000 bp. Scorable bands were produced from each primer ranged from 1 (URP2F) to 7 (URP25F) bands (Table 6).

Table 6
DAMD – DNA banding profiles of DNA samples obtained from different treatments of *Ficus carica* var. Black Jack.

| Primers | Mother plant | *in vitro* control | W | B | R | BR | EX_N | EX_BR | No. of monomorphic Bands | No. of polymorphic Bands | SI Index | Length of amplified DNA fragments (bp) |
|---------|--------------|---------------------|---|---|---|----|------|------|--------------------------|--------------------------|----------|--------------------------------------|
| 6_2H_t  | 2            | 2                   | 2 | 2 | 2 | 2  | 2    | 2    | 0                        | 1.0                      | 500–700   |                                      |
| FV11e8_C| 2            | 2                   | 2 | 2 | 2 | 2  | 2    | 2    | 0                        | 1.0                      | 500–600   |                                      |
| HVA     | 3            | 3                   | 3 | 3 | 3 | 3  | 3    | 3    | 0                        | 1.0                      | 400–1000  |                                      |
| HVR     | 4            | 4                   | 4 | 4 | 4 | 4  | 4    | 4    | 0                        | 1.0                      | 150–400   |                                      |
| URP2F   | 1            | 1                   | 1 | 1 | 1 | 1  | 1    | 1    | 0                        | 1.0                      | 400–500   |                                      |
| URP25F  | 7            | 7                   | 7 | 7 | 7 | 7  | 7    | 7    | 0                        | 1.0                      | 150–1000  |                                      |
| UBC861  | 4            | 4                   | 4 | 4 | 4 | 4  | 4    | 4    | 0                        | 1.0                      | 250–650   |                                      |
| Total   | 23           | 23                  | 23| 23| 23| 23 | 23   | 23   | 0                        | -                        | -         |                                      |
Comparing banding profiles generated using the DAMD – DNA primer it can be determined that genetic stability between the mother plant, *ex vitro*, and the *in vitro* regenerated plants from four different LED treatments is maintained. All the bands acquired using DAMD primers were monomorphic. Thus, these results indicate that there is 0 % polymorphism and 100 % monomorphism (Table 7). Thus the plant genetic stability of *Ficus carica* var. Black Jack is maintained throughout the regeneration experiments as indicated by the SI value of 1.0 as indicated in Table 6.

### Table 7

Monomorphism and polymorphism percentages obtained among the *in vitro* and *ex vitro* plants of *Ficus carica* var. Black Jack.

| Molecular marker | No. of amplicons | No. of monomorphic bands | No. of polymorphic bands | Monomorphism (%) | Polymorphism (%) |
|------------------|------------------|---------------------------|--------------------------|------------------|-----------------|
| ISSR             | 188              | 184                       | 4                        | 97.87            | 2.13            |
| DAMD             | 184              | 184                       | 0                        | 100              | 0               |

### 4. Discussion

ISSR and DAMD markers have been used for the present study to determine the genetic stability among the mother plant and all the *in vitro* plants grown under different light treatments.

Genetic stability or diversity among different plant species is studied using different molecular markers such as RAPD, ISSR, DAMD, SSR, and minisatellite DNA. Assessment of efficiency for such markers was performed using 25 varieties of *Musa acuminata* (Banana). Comparative analysis for testing the polymorphism among the banana varieties using RAPD, ISSR, and DAMD showed that ISSR markers were highly efficient in producing polymorphic bands showing 90.06 % of polymorphism. It was concluded that ISSR and DAMD markers span only selected repeats on the DNA, which allows these markers to competently detect differences in the amplicons (Lamare and Rao, 2015). Genetic stability among Prickly pear cultivars (*Opuntia ficus-indica* L.) was also studied using RAPD markers. Out of 87 amplicons, only 82 bands were monomorphic produced, and overall 91 % of genetic similarity among the plantlets (Zoghlami et al., 2012).

Commercial application of micropropagation on woody plant species can be considered to be successful only when genetic stability is maintained among the plants. Many factors can contribute to the molecular level alterations among the tissue culture plants. The use of PGRs, culture conditions such as light and humidity can initiate genetic instability among the *in vitro* plants. Morphological, cytological, and biochemical changes occurring in the plants can be an indicator of genetic alterations. Thus the molecular assessment of the tissue cultured plants during their juvenile in field stages is crucial. The study of somaclonal variation also allows for the improvement in micropropagation techniques intending to produce genetically identical plants (Leva et al., 2012).

Morphogenetic processes among plants such as stem and root elongation, leaf expansion, biochemical pathways, and metabolisms are greatly affected by the availability of quantity and quality of light. Morphological effects on the *in vitro* growing plants can occur based on the availability of the PAR (Photosynthetically active radiation) (Olle and Viršile, 2013; Mills and Dunn, 2016; Monostori et al., 2018). LED spectra are used for micropropagation of plants to enhance the cellular level properties of the plants. LED differs from the normal light in the sense that LEDs provide a directed flow of the required red and blue photons to the growing cultures. The application of red and blue LEDs has shown efficacy in the yields of many plants (Olle and Viršile, 2013; Borowski et al., 2015; Park and Runkle, 2018). Changes in the photosynthetic parameters and yields of the plants can be attributed to the genetic level changes occurring among the plants (Cioć et al., 2018). Different LEDs were used for the study of growth and yield patterns in a wheat plant. Genes regulating the biosynthetic pathway, for the production of phenylpropanoids in wheat plants growing under white LED were identified using PCR technique. Short sequence primers and qRT-PCR were used to analyse the gene
expression in the *in vitro* plants. It was reported that TaPAL3, TaPAL4, and TaDFR are potential genes that upregulate the production of phenylpropanoids (Cuong et al., 2019).

Alterations occurring in the DNA are due to somaclonal variations. Such changes occurring on the DNA sequences can be easily expressed in PCR analysis which is known as polymorphism. DNA analysis using markers such as RAPD, SSR, ISSR, and DAMD is very commonly used for the detection of polymorphism occurring in any plant species (Devi et al. 2014; Singh et al. 2014; Meena et al. 2019). Genetic diversity was studied for different varieties of date palm using ISSR and DAMD markers. Out of the 113 fragments acquired from PCR, it was shown that 99 were polymorphic and the balance of 14 was monomorphic. This indicated 85.45 % polymorphisms detected in the different cultivars of date palm (Purayil et al., 2018). Similar polymorphism studies were also performed on coniferous plants like Douglas Fir and Sugi, and melon species using ISSR and RAPD markers showed higher percentages of genetic variation (Tsumura et al. 1996; Daryono et al. 2019). Studies have also been performed to test the genetic stability among the plants which undergo tissue culture experiments. Genetic stability assessment of the micropropagated plants of wasabi plant and London plane tree (*Platanus acerifolia*) have revealed that the plantlets are genetically stable (Huang et al. 2009; Matsumoto et al. 2013).

Monomorphism and polymorphism in the DNA fragments can be detected using the concept of similarity index. It uses a mathematical equation to find the relationship between the DNA fragments acquired from PCR and separated using agarose gel electrophoresis. Analysis of polymorphism observed in a single primer was performed using a correlation table. This table relates the similarity index of each individual from the selected population to each other and calculates the similarity index quotient (Huijiang et al., 2005). Molecular analysis was performed on 194 varieties of figs (*Ficus carica* L.) to detect genetic diversity among different germplasm accessions. Genetic diversity among four types of figs such as Common, san Pedro, Smyrna, and Caprifigs was studied using microsatellite markers. Sixteen (16) chosen microsatellite markers showed substantial polymorphism. The heterozygous alleles on different loci indicated exhibited genetic diversity among the cultivars (Aradhya et al. 2010).

Genetic similarity analysis performed using the range of bands acquired from the PCR analysis for both ISSR and DAMD primers shows that all plants are genetically similar to each other. This indicates that throughout the experiment, there is no occurrence of somaclonal variations. However, only primer UBC 840 (Table 4) produced a single polymorphic band with a similarity index of 0.95. This result is due to the presence of an extra polymorphic band in the samples. Such a kind of polymorphism is still considered negligible owing to the possibility of error dynamics in the PCR process.

For *in vitro* generated *Opuntia ficus-indica* L. cultivars, genetic stability assessment using RADP markers presented with a low percentage of polymorphism (2.79%) among the *in vitro* cultures of over 5 years. Thus it was reported that genotypes showing the similarity index percentage of more than 90% are genetically close to each other (Zoghiami et al., 2012). For the different treatments of *Ficus carica* var. Black Jack, polymorphism percentage obtained at 2.12% using ISSR marker. Therefore, a higher percentage of monomorphic bands (97.87%) proves genetic stability among the plants (Table 7). Thus the plant genetic stability of *Ficus carica* var. Black Jack is maintained throughout the regeneration experiments.

### 5. Conclusions

Assessment of genetic stability among the regenerated plantlets and *ex vitro* plants of *Ficus carica* var. Black Jack was successfully performed using ISSR and DAMD molecular markers. Banding profile obtained using DAMD markers showed 100% monomorphism. The banding profile obtained from ISSR markers showed 97.87% of monomorphism with 2.13% of polymorphism. The average genetic similarity index was 0.94 among all the treatments of *Ficus carica* var. Black Jack. Thus the *in vitro* and *ex vitro* plants of *Ficus carica* var. Black Jack was found to be genetically stable.

### Abbreviations

BAP, 6-Benzylaminopurine; DAMD, directed amplification of minisatellite DNA; DNA, deoxyribonucleic acid; IAA, Indole-3-acetic acid; ISSR, inter simple sequence repeats; LED, light-emitting diode; PCR, polymerase chain reaction; PGR, plant growth regulators; WPM, woody plant medium
Declarations

Author contributions

Ankita and Sreeramanan designed and conducted the experiments, analysed the data, and wrote the manuscript. Sreeramanan and Chew Bee Lynn conceptualised and supervised the research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Arrangement of samples in wells of the agarose gel for electrophoresis. Labelling is done as follows mother plant [M], in vitro plant under fluorescent white light (60 μmol.m-2.s-1) [C], white LED [W], blue LED [B], red LED [R], blue + red LED [BR], ex vitro acclimatized plants from normal white light [EXN] and ex vitro acclimatized plants from blue + red (440nm + 660nm) [EXBR]
Figure 2

ISSR – DNA marker results (seven markers) for DNA samples obtained from the samples of Ficus carica var. Black Jack. The primers represented in each gel are, (A) N6; (B) N7; (C) N8; (D) UBC840; (E) UBC811; (F) UBC818 and (G) UBC864.
DAMD – DNA marker results (seven markers) for DNA samples obtained from the samples of Ficus carica var. Black Jack. The primers represented in each gel are, (A) 6_2H_t; (B) FV11e8_C; (C) HVA; (D) HVR; (E) URP2F; (F) URP25F and (G) UBC861