Evaluation of genetic homogeneity of *Jatropha curcas* L. hybrid at an early stage of shoot bud formation from petioles using ISSR marker

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**ABSTRACT**

Genetic homogeneity is known to be the most important prerequisite in the micropropagation of *Jatropha curcas* L. to produce true-to-type plants. The detection of genetic homogeneity in clonal micropropagation for elite plants at an early stage is required, to avoid any increase in variation in the next stage of micropropagation. The genetic homogeneity was assessed during shoot bud formation from petiole explants of *J. curcas* (P1 × P3) hybrid with different concentrations of thidiazuron (TDZ) in a range of 0.5–4.0 mg/L using inter simple sequence repeat (ISSR) markers. Out of 23 ISSR primers, 16 primers produced clear, distinct and reproducible bands. A total of 96 bands, ranging in size from 100 to 1013 bp were generated. Based on the band data, a total of 94 bands were monomorphic (98%) and two bands were polymorphic (2%). All banding patterns from the shoot buds induced by 0.5, 1.0 and 2.0 mg/L TDZ were monomorphic, but 4.0 mg/L gave 2% polymorphism. These findings indicated that concentrations of 0.5, 1.0 and 2 mg/L TDZ did not trigger any somaclonal variation and could, therefore, be considered suitable for application in clonal micropropagation of *J. curcas* hybrid using petioles as explant material.

**INTRODUCTION**

*Jatropha curcas* L. has many common names in different countries worldwide, where it is known as purging or physic nut (English), Jarak pagar (Malaysian and Indonesian) and Ratan-jayot (Bengali). *J. curcas* is significantly important for its value in alternative medicine, and as a renewable energy source, due to its superior performance and environmental features [1]. Recently, Jatropha oil has been used as an aviation biofuel due to its high stability even at low temperatures [2]. Carbon dioxide and smoke emissions from Jatropha oil are also lower than from petroleum diesel, thus making Jatropha oil an eco-friendly source [3]. Several therapeutic activities have also been reported for Jatropha, since it contains many phenolic compounds, which can have antiviral [4], anticancer, anti-inflammatory and antioxidant properties [5].

Major limitations exist for the large-scale cultivation of *J. curcas* as an energy crop, particularly due to the unavailability of seeds. This might be due to problems during the establishment of nurseries and to inconsistent seed yield because of the heterogeneous nature of the plant material [6]. Micropropagation is the best technique to produce a large amount planting material with desired traits in a short time and small space. The micropropagation of *J. curcas* has been reported from various explants, such as nodes [7], shoot tips [8], petioles [9,10], leaves [11], hypocotyls [12] and embryos [13]. The establishment of Jatropha plants from various explants will enhance the production of plant material from the elite mother plant. In such cases, Jatropha seeds would not be used as planting material, since these are used for oil production.

The *in vitro* propagation of *J. curcas* using thidiazuron (TDZ) as the basic plant growth regulator has been reported by some researchers [14–16]. TDZ is a substituted phenylurea compound that has been shown to possess both auxin- and cytokinin-like effects, although it is structurally different from either auxins or purine-based cytokinins [17].

Genetic stability is the most crucial aspect to be considered when generating planting material via micropropagation, to maintain the superior quality of the donor plants. According to Pierik [18], the petiole is genetically stable to be used as explant material, as it originates from somatic cells. Several studies on genetic stability following regeneration from petioles in various plant species have shown a high degree of monomorphism [19,20]. To the best of our knowledge, no report exists concerning the genetic stability of plants derived from
petioles of *J. curcas* (P1 × P3) hybrid. According to Islam [21], the *J. curcas* (P1 × P3) hybrid offers advantages over other Jatropha plants in terms of early fruiting and a high seed yield per plant. Therefore, in this study, the clonal micropropagation for this hybrid needs to be established for large-scale production. During the clonal production of elite plants, somaclonal variation is known to reduce some of their commercial value. Thus, it is important to determine any variation that occurs at an early stage of *in vitro* development [22].

Some molecular methods have been previously applied for genetic stability studies in *J. curcas*, such as the use of amplified fragment length polymorphisms (AFLPs) [23], random amplified polymorphic DNA (RAPD) [24] and flow cytometry [25]. The use of inter simple sequence repeats (ISSRs) offers unique advantages over other molecular markers, since their application does not require any genomic information of the target species, it only requires a small amount of template DNA, is rapidly performed [26] and is highly efficient in detecting highly polymorphic DNA among Jatropha genotypes [27,28]. An ISSR marker system has been successfully used to detect somaclonal variation in *J. curcas* [25,29,30].

This study was conducted to determine the genetic stability of *in vitro*-raised shoot buds of petiole explants of *J. curcas* hybrid (P1 × P3) induced using various concentrations of TDZ (0.5, 1.0, 2.0 and 4.0 mg/L).

**Materials and methods**

**Plant material and explant preparation**

Petioles from mature plants were collected from a three-year-old *J. curcas* hybrid (P1 × P3) in Living Lab Energy and Future Crops (UKM Kuala Pilah). The petioles were washed under running tap water for 15 min and were rinsed with two drops of teepol. The petioles then were soaked in 3 mL systemic fungicide Myzim in 500 mL of sterile distilled water for 30 min. The petioles were rinsed three times in sterile distilled water containing one drop of Tween-20 and were then surface-sterilized with 70% ethanol for 1 min, rinsed three times and treated 5 min with 0.1% (w/v) mercuric chloride (HgCl₂) followed by five rinses with sterile distilled water. The petioles then were cut into 0.5–1.0 cm sections and the edges were removed for shoot induction.

**Induction of shoots and culture conditions**

The petiole explants were placed horizontally on Murashige and Skoog (MS) medium [31]. Shoot buds were induced using different concentrations of TDZ at 0.5, 1.0, 2.0 or 4.0 mg/L on MS medium. All the samples were incubated at 25 ± 2 °C with a 16 h light/8 h dark photoperiod at a light intensity of 35–40 μmol m⁻² s⁻¹ (cool white fluorescent tubes) [16]. The formation of shoot buds was observed within 7–8 weeks of culture. Thirty explants were used for each concentration and the experiments were performed in triplicate.

**DNA extraction and DNA quantification**

Genomic DNA was extracted from leaves of hybrid mother plants (P1 × P3) and regenerated shoot buds using the InnuPREP plant DNA kit supplied by Analytic Jena (Jena, Germany). The plant materials were ground in liquid nitrogen, which helped to freeze the plant material to increase the surface area for DNA extraction. DNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA, United States) at 260 nm.

**Primers**

A total of 23 ISSR primers (13 from the UBC primer set and 10 primers from Sigma were tested on DNA of *J. curcas* (P1 × P3) hybrid mother plants and shoot buds induced *in vitro* (Table 1).

**Polymerase chain reaction (PCR) amplification conditions**

The PCR amplification was performed in a total reaction volume of 50 μL which contained 25 μL master mix (Bioline, London, UK), 2 μL primers (1 μmol/L), 3 μL DNA (200 μg/μL) and 20 μL Nanopure water. The DNA amplification

| Serial No. | ISSR primer | Sequence |
|------------|-------------|----------|
| 1          | UBC807      | 5’ AGAGAGAGAGAGAGAGAGT 3’ |
| 2          | UBC834      | 5’ AGAGAGAGAGAGAGAGAGTT 3’ |
| 3          | UBC836      | 5’ AGAGAGAGAGAGAGAGAGCA 3’ |
| 4          | UBC848      | 5’ ACACACACACACACACAG 3’ |
| 5          | UBC856      | 5’ ACACACACACACACACCA 3’ |
| 6          | UBC873      | 5’ GACAGAGAGAGAGAGAGA 3’ |
| 7          | UBC889      | 5’ ACACACACACACACACAC 3’ |
| 8          | UBC808      | 5’ AGAGAGAGAGAGAGAGG 3’ |
| 9          | UBC809      | 5’ AGAGAGAGAGAGAGAGG 3’ |
| 10         | UBC810      | 5’ ACACACACACACACACAT 3’ |
| 11         | UBC811      | 5’ GAGAGAGAGAGAGAGAC 3’ |
| 12         | UBC814      | 5’ CTCTCTCTCTCTCTCTCT 3’ |
| 13         | UBC815      | 5’ CTCTCTCTCTCTCTCTCTG 3’ |
| 14         | 11          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 15         | 12          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 16         | 13          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 17         | 14          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 18         | 15          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 19         | 16          | 5’ GAGAGAGAGAGAGAGAGG 3’ |
| 20         | 17          | 5’ CTCTCTCTCTCTCTCTCTG 3’ |
| 21         | 18          | 5’ ACACACACACACACAC 3’ |
| 22         | 19          | 5’ ACACACACACACACACAC 3’ |
| 23         | 110         | 5’ GAGAGAGAGAGAGAGG 3’ |
was performed at 95 °C for 3 min for initial denaturation, followed by 34 cycles at 95 °C for 3 s, 50 °C for 1 min and 72 °C for 1 min for extension, followed by a final step extension at 72 °C for 10 min. All reactions were carried out in a PCR thermocycler (Eppendorf Mastercycler DNA engine, Hamburg, Germany). After amplification, each PCR reaction was analysed by electrophoresis in a 1% Tris-acetate-EDTA (ethylenediaminetetraacetic acid) buffer agarose gel, stained using Gelred and visualized by a Gel Documentation System (Bio-Rad).

**Data scoring and analysis**

Only distinct, reproducible and well-resolved fragments ranging from 100 to 1000 bp were considered in the analysis. These bands were scored either as present (1) or absent (0) for each of the ISSR markers within the shoot buds induced by different concentrations of TDZ and in the mother plants. The presence of DNA bands at a low intensity that could not be readily distinguished as present or absent was considered ambiguous markers and were not scored. The size of each band was estimated using a DNA ladder (HyperLadder 100 bp Plus, Bioline, London, UK). Data are presented as mean values with standard deviation (±SD) of 30 explants per treatment in three independent experiments.

**Results and discussion**

This study was conducted to screen for genetic variation at an early stage of shoot bud formation from tissue culture of *J. curcas* that used matured petioles as a starting material. According to Liu et al. [10], petioles that are too young or too old are not suitable as starting material, since they have low regeneration frequency. Furthermore, petioles that are too young are susceptible to sterilization agents and lead to the death of the tissue. Therefore, mature petioles were selected as the best explant material for the study. The genetic variation was determined by employing the ISSR-PCR assay. Shoot buds were induced by all concentrations of TDZ tested, ranging from 0.5 to 4.0 mg/L (Figure 1). A concentration of 1.0 mg/L TDZ gave the highest number of shoot buds (*p < 0.01*), compared to other concentrations of TDZ. These results are in agreement with the study by Zhang et al. [15], where the same concentration of 1.0 mg/L of TDZ is also reported to give highest shoot bud formation (Table 2).

All the primers (Table 3) applied in this study were selected according to Grativol et al. [28] and Kumar et al. [29]. In total, 23 ISSR primers were screened for amplified bands, but 16 primers were chosen for further analysis, as they generated 96 clear and reproducible amplification products. Thus, on average, six bands were amplified per primer. The primer UBC 834 amplified the maximum number of 14 bands, whereas UBC 811 amplified the lowest number of three bands (Table 3). All the primers amplified scorable bands that ranged in size between 100 bp and 1013 kb.

**Table 2.** Effect of different concentrations of TDZ on the percentage of shoot bud induction.

| TDZ (mg/L) | Shoot bud induction (%) |
|-----------|-------------------------|
| 0.5       | 7.64 ± 1.62*            |
| 1         | 50.7 ± 1.32**           |
| 2         | 10.7 ± 1.57**           |
| 4         | 15.5 ± 1.40**           |

Note: Values are means ± SD of 30 explants per treatment in three independent experiments.

*Significant difference at *p* < 0.05.

**Table 3.** Performance of different ISSR primers for the detection of genetic stability in sample shoot buds of *J. curcas* (*P1 × P3*) hybrid.

| Serial No. | Primer code | Number of amplified fragments | Number of polymorphic bands | Range of amplification (bp) |
|------------|-------------|-------------------------------|----------------------------|--------------------------|
| 1          | UBC834      | 14                            | 0                          | 100–900                  |
| 2          | UBC889      | 7                             | 0                          | 300–900                  |
| 3          | UBC807      | 6                             | 0                          | 100–400                  |
| 4          | UBC836      | 8                             | 0                          | 200–1013                 |
| 5          | UBC848      | 5                             | 0                          | 500–1013                 |
| 6          | UBC856      | 5                             | 2                          | 100–1013                 |
| 7          | UBC873      | 7                             | 0                          | 200–1013                 |
| 8          | UBC808      | –                             | –                          | –                        |
| 9          | UBC809      | –                             | –                          | –                        |
| 10         | UBC810      | –                             | –                          | –                        |
| 11         | UBC811      | 3                             | 0                          | 100–300                  |
| 12         | UBC814      | –                             | –                          | –                        |
| 13         | UBC815      | 3                             | 0                          | 100–400                  |
| 14         | I1          | 6                             | 0                          | 300–900                  |
| 15         | I2          | 6                             | 0                          | 300–900                  |
| 16         | I3          | 4                             | 0                          | 100–300                  |
| 17         | I4          | –                             | –                          | –                        |
| 18         | I5          | 8                             | 0                          | 100–900                  |
| 19         | I6          | –                             | –                          | –                        |
| 20         | I7          | 5                             | 0                          | 100–400                  |
| 21         | I8          | 4                             | 0                          | 200–500                  |
| 22         | I9          | 5                             | 0                          | 100–400                  |
| 23         | I10         | –                             | –                          | –                        |
| Total      |             | 96                            | 2                          | –                        |
In this study, 94 bands were monomorphic (98%) and two bands were polymorphic (2%), giving a total of 96 bands. The amplification of monomorphic bands from shoot buds of *J. curcas* was obtained using the primers I5 and UBC 889 and representative gel profiles are shown in Figure 2. Fifteen out of 16 primers produced monomorphic bands, i.e. ones that are present in all individuals and are inherited by the subsequent generation, confirming the genetic stability of the shoot buds produced by TDZ in vitro. The total number of 96 marker bands amplified during the assay in this study is sufficient to detect any somaclonal variation. This is comparable to the 40, 45 and 51 bands amplified in other previous studies for various plant species employing ISSR-based markers to evaluate genetic stability [32–34]. The ISSR analysis in this study showed that the shoot buds generated from tissue culture were completely uniform, suggesting a high level of genetic homogeneity among them. This indicated that petioles represent an explant source that can produce true-to-type plants in *J. curcas* micropropagation, instead of shoot tips and nodal explants. According to Pierik [18], plants raised from petioles are more resistant to genetic variation because they originate from somatic tissue. For example, the use of petioles of broccoli as explant material gives a high degree of monomorphism [20]. In *J. curcas*, a few studies have used petioles as explant material for micropropagation [9,10,16]. However, to the best of our knowledge, there are no reports on the genetic stability in petiole explants of *J. curcas*. In this study, genetic variation was detected in the shoot buds at the induction stage, because during this early stage, the cells are in the adaptation period. During this period, the plant material experiences a high level of stress, and variation might be induced to occur. No detected variation during the early stage of shoot bud formation indicates a reduced probability of somaclonal variation. According to Sharma et al. [6], the screening of somaclonal variation in micropropagated plantlets at an early stage of development is necessary to avoid an increase in variation in the future.

The role of growth hormones is one factor that can cause genetic changes in vitro, and when applied at higher concentrations, hormones can cause genetic instability, or so-called spontaneous variation [35]. For example, a high concentration of cytokinin (6 mg/L BAP) applied to the initial culture can lead to somaclonal variation in *J. curcas* using axillary buds as explant material [6]. Some studies have reported that the application of TDZ at high concentrations leads to phenotypic abnormalities and to the inhibition of shoot regeneration and root development in *J. curcas* [15], as well as in other plants [36]. This is probably related to the somaclonal variation (genetic instability) that was observed following exposure to a high TDZ concentration of 4.0 mg/L, at which genetic variation occurred in this study (Figure 3).

![Figure 2. Monomorphic bands of ISSR products amplified from in vitro-raised shoot buds treated with different concentrations of TDZ (0.5, 1.0, 2.0 and 4.0 mg/L) and from mother plants of *J. curcas* using ISSR 5 (A) and UBC 889 (B) primers. Lane M, molecular size marker; MP, mother plant.](image)

![Figure 3. Polymorphic bands of ISSR products amplified from in vitro-raised shoot buds treated with different concentrations of TDZ (0.5, 1.0, 2.0 and 4.0 mg/L) and from mother plants of *J. curcas* using UBC 856 ISSR primers. Lane M, molecular size marker; MP, mother plant. Note: Polymorphic bands are shown by an arrow at 4 mg/L TDZ.](image)
In this study, lower TDZ concentrations (0.5, 1.0 and 2.0 mg/L) did not induce somaclonal variation. This is consistent with the results for two in vitro-derived orchid species that gave 100% monomorphism [37] and 97% genetic fidelity at low concentrations of TDZ [36]. Overall, the results from the present study indicated that concentrations of TDZ in the range of 0.5–2.0 mg/L did not trigger any somaclonal variation and could, therefore, be considered suitable for application in clonal micropropagation of *J. curcas* hybrid using petioles as explant material.

### Conclusions

This study demonstrated that the use of TDZ at concentrations of 0.5, 1.0 and 2.0 mg/L is suitable for the induction of shoot buds from petioles of *J. curcas* and no genetic variation was induced. However, the application of 4.0 mg/L can produce a small amount of genetic variation (2%). This is of practical importance, since the early detection of genetic variation is useful and important to generate true-to-type plants. The early detection of somaclonal variation using ISSR analysis is effective for micropropagation techniques in any plant species.

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### Disclosure statement

No potential conflict of interest was reported by the authors.

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