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# ANALYSIS OF GENETIC STABILITY OF MICROPROPAGATED *OCHRADENUS BACCATUS*

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**ABSTRACT:**

*Ochradenus baccatus* (Del.), an important medicinal plant, belongs to the family Resedacea. Presently this is an endangered species therefore non-conventional methods for their propagation are requested to prevent its eradication. We developed a micropropagation method for mass multiplication of *O. baccatus*. Explants collected from South Sinai were used to establish cultures of *O. baccatus*. Surface sterilized nodal shoot segments and shoot tips were used for micropropagation by culturing on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of BA and 2ip. Stem node sections were superior in enhancing formation of sub axillary shoots on MS medium supplemented with 1.0 mg/l BA + 1.0 mg/l 2ip. The multiplied shoots were then rooted on WPM supplemented with 1.0 mg/l IBA. The plantlets were acclimatized *ex vitro*. Genetic stability of plants produced *in vitro* was evaluated using random amplified polymorphic DNA (RAPD) analysis. Plants produced under different concentrations of hormones, mother plant and the plants grown *ex vitro* were analyzed by RAPD using 8 random 10-mer DNA primers. Results showed that 73 RAPD bands were obtained which gave 39.72 % polymorphism. The production of variant plants is not good for commercial propagation. However, it could be minimized by reducing the subculture cycle or by reducing hormone level in media. Additionally, this variability could be used for further improvement and selection of new cultivars and is an important source of variability to be exploited.

## INTRODUCTION:

*Ochradenus baccatus* (Del.) belongs to the family Resedacea. The plant has been known for its medicinal uses. This species has previously been shown to reduce blood cholesterol level in rats (Barakat *et al*., 1991) and demonstrated strong growth inhibition of the malaria parasite P*lasmodium falciparum*. (Sathiyamoorthy *et al*., 1999). This plant species is distributed in deserts of South Sinai. Use of this species as a fodder plant led to difficulties in its vegetative propagation or in obtaining its seeds from their native areas because of overgrazing, which make this plant endangered. There is a need for developing alternative methods for quick and efficient propagation. Plant tissue culture as a technology for *ex situ* multiplication is fast, uses small amounts of shoots and may succeed when other methods fail (Edson *et al*., 1997). In recent years, there has been an increased interest in *in vitro* culture technique which offer available tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora and Bhojwani, 1989).

Occurrence of somaclonal variation is a potential drawback when the propagation

of an elite genotype is intended, where clonal fidelity is required to maintain the advantages of desired elite genotypes (Rahman and Rajora, 2001). On the other hand stable somaclonal variations of specific type may be advantageous for the improvement of certain traits (Antonetti and Pinon, 1993; Karp 1995, Jain *et al*., 1998). It has been reported that *in vitro* cultured plantlets might exhibit somaclonal variation (D'Amato, 1978; Skirvin, 1978; Larkin and Scowcroft, 1981). As gross morphological variations are expected to occur at much lower frequency than cryptic variation (Evans *et al*., 1984), the absence of visible variations does not preclude the absence of all variation among micropropagated progeny. Allozyme markers can be used for examining cryptic somaclonal variation but these markers are limited by both number and amount of polymorphism and their developmentally regulated expression. DNA markers are more attractive means for examining genetic similarity / dissimilarity since they are not developmentally regulated.

Recently, random amplified polymorphic DNA markers (RAPDs) have been applied for characterization of micropropagated forest trees (Isabel *et al*., 1993, Rani *et al*., 1995, Barrett *et al*., 1997, Rahman and Rajora, 2001). These results showed that RAPD markers can be used to obtain rapid information about genetic similarities / dissimilarities in micropropagules. RAPD markers (William *et al*., 1990) were employed to determine the genetic homogeneity/ somaclonal variation of the micropropagated plants of *Ochradenus baccatus*.

Micropropagated plants from the cultures of preformed structures such as shoot tips and axillary buds have been reported to maintain clonal fidelity (Ahuja, 1987, Wang and Charles, 1991, Ostry *et al*., 1994) but there is still a possibility of generating somaclonal variants employing this method (Rani and Raina, 2000).

The aim of this work was to establish a suitable method for the propagation of *Ochradenus baccatus via* micropropagation technique and study the genetic stability of the propagated plants using RAPD analysis.

## MATERIALS AND METHODS

### Micropropagation experiments

**Explant preparation:** Explants were collected in winter from Sedr Alhetan, South Sinai. Actively growing shoots with terminal buds were collected, moistened and wrapped. Explants were washed under running tap water for 20 – 30 minutes for stem node sections and 10 – 15 minutes for shoot tips.

**Culture medium and conditions:** The culture medium used for the present work was MS medium (Murashige and Skoog, 1962) supplemented with 100mg/l *myo*-inositol and 3% sucrose.The medium was further supplemented with different combination of 0.0, 0.1 mg/l NAA and 0.0, 0.25 and 0.5 mg/l 2ip. The pH of the medium was adjusted to 5.7 - 5.8 before the addition of 2.5 g/L phytagel.

The sterilized explants were cultured on the media under complete aseptic conditions in the Laminar Air Flow Hood and placed in incubation room at 26 $^{+}_{-}$2 $C^{o}$ under a photoperiod of 16 hour. After 4 weeks, the shoots were counted and their lengths were measured.

**Multiplication of shoot cultures:** Shoots were sectioned into two node pieces after removing the leaves. The nodal segments containing the axillary buds were cultured in MS supplemented with 0.0, 0.5, 1.0, 1.5, 2.0 mg/l of BA and 2ip in all combinations for further multiplication. Shoots were subcultures after every 4 weeks on the same media.

**Rooting of shoots:** The multiplied shoots were induced for rooting in four different rooting media; WPM without growth regulators, WPM supplemented with 0.5 and 1.0 mg/L IBA. WPM supplemented with 0.5 mg/l IBA and 0.5 mg/L NAA. After 4 weeks, number of roots per shoot and root length were measured.

**Acclimatization and transfer of plantlets to soil:** The rooted plantlets were removed from the test tubes, washed gently under tap water to minimize injury and to free the roots from phytagel. The plantlets were transferred to plastic pots containing a mixture of peatmoss and sand (1:1) then enveloped in polyethylene bags and incubated under 16 hours photo period at 25 ±1C$^{o}$ in growth cabinets.

After 4 weeks polyethylene bags were completely removed and plantlets were maintained under greenhouse conditions.

**Genetic stability studies**

**DNA extraction:** Leaves of *Ochradenus baccatus* plant were kept frozen at -80 C$^{o}$ as mother samples for extracting DNA. The DNA was extracted followed method as described by Pirttila *et. al*., (2001). For this purpose Leaf sample were ground to a fine powder in liquid nitrogen using pre-cooled pestle and mortar and 0.1 g. of the powder was transferred to a pre-warmed (65°C) mixture of 350µl of extraction buffer immediately added before use and 350µl of 8M LiCl in a micro centrifuge tube. The genomic DNA was resuspended in 10-50µl deionized $H_2O$. The nucleic acid concentration was determined by spectrophotometer at 260 nm.

**PCR amplification:** PCR reactions were conducted using arbitrary 10 mer primers synthesized at Operon Technologies Inc.

**Table-1:** List of Operon RAPD primers with their nucleotide sequences used for *Ochradenus baccatus* genome finger printing

| Description | Sequence |
|---|---|
| OPA-02 | 5'-TGCCGAGCTG-3' |
| OPA-17 | 5'-GACCGCTTGT-3' |
| OPA-13 | 5'-CAGCACCCAC-3' |
| OPB-13 | 5'-TTCCCCCGCT-3' |
| OPB-18 | 5'-CCACAGCAGT-3' |
| OPZ-13 | 5'-GACTAAGCCC-3' |
| OPZ-12 | 5'-TCAACGGGAC-3' |
| OPA-11 | 5'-CAATCGCCGT-3' |

PCR conditions were carried out in a reaction volume of 25µl containing 5µM/µl Taq DNA polymerase, 10X buffer with 15 mM $MgCl_2$, 200 µM of each dNTP, 10mM of primer (Operon Technologies Inc.), 50 ng of genomic DNA. Amplification was performed in Perkin Elmer Gene Amp 9700 PCR system version 3.03 with initial denaturation cycle at 94 Cº for 4 min. followed by 45 cycles consisting of 94 Cº for 1.5 min., 37.5Cº for 1.5 min., 72Cº for 2.5 and a final extension cycle at72 Cº for 7 min. Amplification products were separated in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and photographed with Bio-Rad Gel Doc. System.

**Data evaluation:** The amplification of DNA samples was repeated thrice and only bands reproducible on several runs were considered for analysis. Each amplified product was scored across all the micro-propagated plants under different hormone concentrations and mother plant of *Ochradenus baccatus* for eight random 10 mer primers. Bands were scored as present (1) or absent (0) for each sample. Co-migrating bands were considered to represent the same locus. Weak and spurious bands were not included in the analysis.

## RESULTS AND DISCUSSION

**Explant sterilization:** The two types of explants were subjected to different concentrations of sodium hypochlorite for sterilization (Table 2). In the case of shoot tips the highest survival percentage was

93.0%, which achieved by using 1.5 % sodium hypochlorite for 15 minutes. While in the case of stem node sections, the highest survival percentage was 100 % by using 15 % of sodium hypochlorite for 10 minutes

**Table-2:** Effect of surface sterilization with different concentrations and durations of sodium hypochlorite (NaOCl) solution on the survival percentage of shoot tip and stem node section explants of *Ochradenus baccatus*.

| Duration (min.) | NaOCl concentrations % for | | Survival % | |
|---|---|---|---|---|
| | Shoot tip | Stem node section | Shoot tip | Stem node section |
| 10 | 1.5 | 10 | 69.0 | 90.00 |
| 15 | 1.5 | 10 | 93.0 | 73.00 |
| 10 | 2.5 | 15 | 71.2 | 100.0 |
| 15 | 2.5 | 15 | 62.0 | 93.33 |
| 10 | 5 | 30 | 48.6 | 71.00 |
| 15 | 5 | 30 | 30.0 | 66.00 |

Despite all treatments used for sterilization, contamination still persists as a difficult problem. This is possibly due to some endogenous microorganisms that are harbored within the explant tissue. Some of these contaminations are slow growing or latent and will not visually apparent for several subcultures (Hu and Wang, 1983)

**Establishment of cultures:** The primary goal of the establishment stage is to obtain a large percentage of explants free from surface pathogens (Murashige, 1974). Factors that affect the success of this stage include choice of explant, elimination of contaminations, and culture conditions, which include ingredients, light, temperature and choice of explant support (Hartmann and Kester, 1983).

**Table-3:** *In vitro* establishment of stem node sections of *Ochradenus baccatus* cultured on MS nutrient medium supplemented with NAA and 2ip.

| Conc. mg/L | | Shoot tips | | | | Stem node sections | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | % of survived explants. | % of explants. forming axillary shoot | Mean no. of shoots | Mean length of shoots | % of survived explants. | % of explants. forming axillary shoot | Mean no. of shoots | Mean length of shoots |
| 0.0 | 0.0 | 100 | 100 | 1 | 1.8 $^{b}$ | 100 | 55.5 | 1.4 $^{c}$ | 0.83$^{a}$ |
| 0.0 | 0.25 | 80 | 87.5 | 1 | 1.7 $^{b}$ | 90 | 100 | 6.2 $^{b}$ | 0.18$^{b}$ |
| 0.1 | 0.25 | 90 | 100 | 1 | 1.8 $^{b}$ | 90 | 100 | 7.2 $^{b}$ | 0.15$^{b}$ |
| 0.0 | 0.5 | 100 | 100 | 1 | 2.5 $^{a}$ | 100 | 100 | 6.8 $^{b}$ | 0.21$^{b}$ |
| 0.1 | 0.5 | 100 | 100 | 1 | 2.1$^{ab}$ | 90 | 100 | 10.4$^{a}$ | 0.26$^{b}$ |

Means followed by the same letter are not significantly different ($p < 0.05$) according to ANOVA and LSD multiple- range test.

In this study different concentrations of zip (0.0, 0.25, 0.5 mg/L) were used in combinations without or with 0.1 mg/L NAA to enhance shoot induction. Results presented in Table-3 showed that, for shoot tip explants there is no significant differences between the five used media in the mean number of shoots, each of them gave only one shoot per shoot tip. It was observed that media contains 0.5mg/L 2ip gave the highest mean length of shoot which was 2.56 cm, the second best length was 2.1 which achieved by using MS medium supplemented with 0.1mg/L NAA and 0.5mg/L 2ip. For stem node sections, the highest percentage of explants forming axillary shoots and means numbers of shoots were obtained in the presence of 2ip. The presence of NAA (0.1 mg/L) and 0.5 mg/L 2ip gave the best mean number of shoots and the MS free medium gave the best mean length of shoot. (Figure 1a, b).

The presences of auxin and cytokinin hormone in the establishment medium are essential for shoots formation (Skirvin, 1981; Cate and Ramula, 1987). However, Butiuc and Deliu (2001) reported that micropropagation of *A. montana* using MS medium supplemental with 2-isopentenyl adenine (2ip), Zeatin and alpha-naphthalene acetic acid (NAA) in different concentrations does not ensure the formulation of a high number of regenerated plants.

**Multiplication of shoot cultures:** Multiplication is a rapid increase of organs, which can ultimately give rise to plant. This increase is achieved by enhancing axillary shoot initiation (Murashige, 1974). This stage is repeated at regular intervals to produce large-scale multiple shoots, which is commercially useful (Smith and Murashige, 1970). Large amount of callus were formed with established explants in the presence of NAA may cause inhibition to further stages. For that reason, we concentrated on using different combinations of both BA and 2ip to enhance initiation and multiplication of shoots and hinder callus formation (Figure 1 a, b). The choice of BA as a cytokinin was because it is effective *in vitro* shoot proliferation and multiplication with many woody species (Murashige, 1974; Sharma *et al*., 1981 and Bennet and Davies, 1986) and the choice of 2ip in combinations with BA was because it is effective in enhancing elongation of axillary shoots.

Our results indicated that the best medium for obtaining the highest mean number of shoots / explants (31.8) were MS supplemented with 1.0 mg/L BA and 1.0 mg/L 2ip (Table 4). This medium gave also 100% survived explants however it was not the best medium for the shoot length (1.01 cm). This decrease in length of shoots may associate with increase in their number. The second best medium, which contained 2.0 mg/L BA and 1.0 mg/l 2.0 gave mean number of shoots (26.8). Media containing 2.0 mg/l 2ip alone gave the best mean length of shoot/explant (1.58 cm), followed by medium containing 1.5 mg/L 2ip and 0.5 mg/l BA which gave shoot length (1.56 cm). Addition of any concentration of BA to media containing 2 mg/L 2ip reduces the mean length of shoot. Increasing or decreasing the concentration of both cytokinin decreased significantly the mean number of axillary shoots per explant. In general supraoptimal cytokinin concentrations may promote the number of shoots produced while delaying their appearance (Webb *et al.*, 1988). Furthermore, high BA levels often produce hyperhydric malformed adventitious shoots which are undesirable for clonal propagation (Tanuwidjaja *et al*., 1998).

**Table-4:** *In vitro* multiplication of *Ochradenus baccatus* cultured on MS nutrient medium supplemented with different concentrations of BA and 2ip.

| No. | Conc. mg/L | | % of survived exp. | % of explants forming shoot | Mean number of shoots / exp. | Mean length of shoot / exp.(cm) |
|---|---|---|---|---|---|---|
| | BA | 2ip | | | | |
| 1 | 0.0 | 0.0 | 66.6 | 60 | 3.0 hi | 0.63 cde |
| 2 | 0.5 | 0.0 | 80 | 66.6 | 3.6 hi | 0 .84 cde |
| 3 | 1.0 | 0.0 | 73.3 | 90.9 | 6.6 ghi | 0.93 cde |
| 4 | 1.5 | 0.0 | 93.7 | 93.3 | 7.6 efghi | 0.5 de |
| 5 | 2.0 | 0.0 | 86.6 | 76.9 | 8.6 fghi | 0.51 de |
| 6 | 0.0 | 0.5 | 100 | 93.3 | 2.0 i | 1.18 abc |
| 7 | 0.5 | 0.5 | 100 | 71.4 | 11.6 defghi | 0.95 cde |
| 8 | 1.0 | 0.5 | 86.6 | 92.3 | 4.8 ghi | 0.81 cde |
| 9 | 1.5 | 0.5 | 93.3 | 85.5 | 18.4 bcdef | 0.62 cde |
| 10 | 2.0 | 0.5 | 100 | 76.9 | 23.2 abc | 0.74 cde |
| 11 | 0.0 | 1.0 | 85.7 | 100 | 19.0 bcde | 1.15 abc |
| 12 | 0.5 | 1.0 | 73.3 | 90.9 | 5.4 ghi | 0.87 cde |
| 13 | 1.0 | 1.0 | 100 | 93.3 | 31.8 a | 1.01 bcd |
| 14 | 1.5 | 1.0 | 85.7 | 83.3 | 17.6 bcdef | 0.98 cd |
| 15 | 2.0 | 1.0 | 85.7 | 75 | 26.8 ab | 0.73 cde |
| 16 | 0.0 | 1.5 | 100 | 64.2 | 15.6 cdefg | 1.14 abc |
| 17 | 0.5 | 1.5 | 80 | 83.3 | 21.6 bcd | 1.56 ab |
| 18 | 1.0 | 1.5 | 66.6 | 90 | 18.4 bcdef | 1.03 bcd |
| 19 | 1.5 | 1.5 | 73.3 | 90.9 | 13.4 cdefgh | 0.88 cde |
| 20 | 2.0 | 1.5 | 100 | 92.3 | 8.0 fghi | 0.52 de |
| 21 | 0.0 | 2.0 | 92.8 | 92.3 | 19.4 bcde | 1.58 a |
| 22 | 0.5 | 2.0 | 93.3 | 71.4 | 12.8 cdefghi | 1.02 bcd |
| 23 | 1.0 | 2.0 | 92.8 | 92.3 | 3.0 hi | 0.75 cde |
| 24 | 1.5 | 2.0 | 100 | 84.6 | 18.0 bcdef | 0.52 de |
| 25 | 2.0 | 2.0 | 66.6 | 87.5 | 3.6 hi | 0.4 e |

Means followed by the same letter are not significantly different ($p < 0.05$) according to ANOVA and LSD multiple- range test.

**Rooting of shoots**

IBA possesses the indole nucleus characteristic of IAA, but has a 4-carbon, rather than a 2-carbon side chain. NAA, on the other hand, possesses a side chain similar to that of IAA but contains a different nucleus (Krishnamoorthy, 1981).

Results in Table-5 indicate that WPM supplemented with 1 mg/L IBA gave the highest percentage of explants forming roots (87.5%), The same media gave the best mean number of roots per explant (3.38 a) and the best mean length of roots (2.9cm). This is in agreement with Jansson and Welander (1999) who reported that shoots of some adult Betula species were rooted on basal WPM with 0.1 mg/L IBA. Also, Bertolucci *et al*. (2000) used WPM in inducing roots in *Tournefortia paniculata*. Optimum rooting response-using IBA has

also been reported for several plant species including *Syzygium alternifolium* (weight) *walp* (Sha Valli Khan *et al*., 1997), *Azadirachta indica* (Eeswara *et al*., 1998), *Melia azedarach* (Thakur *et al*., 1998), *Anacardium occidentale* (Bogetti *et al*., 1999) and *Dendrocalamus asper* (Arya *et al*., 1999).

**Table-5:** Rooting of *in vitro* multiplied shoots of Ochradenus *baccatus* culture on WPM supplemented with different concentrations of NAA and IBA.

| Media | % of explants forming roots | Mean no. of roots / exp. | Mean length of roots/exp. (cm) |
|---|---|---|---|
| WPM | 33.3 | 1.0 $^{b}$ | 0.85 $^{b}$ |
| WPM + 0.5 mg/L IBA | 71.4 | 2.25 $^{b}$ | 1.83 $^{b}$ |
| WPM + 1.0 mg/L IBA | 87.5 | 3.38 $^{a}$ | 2.9 $^{a}$ |
| WPM + 0.5 mg/L IBA + 0.5 mg/L NAA | 57.1 | 0.88 $^{b}$ | 0.85 $^{b}$ |

Means followed by the same letter are not significantly different ($p < 0.05$) according to ANOVA and LSD multiple- range test.

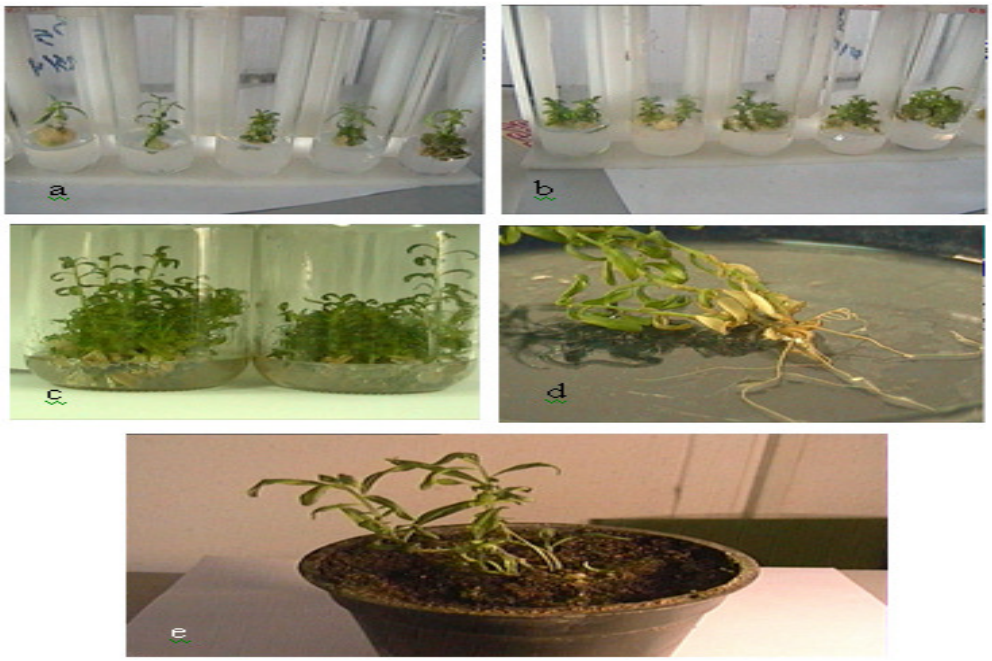


**Figure-1:** Micropropagation of *Ochradenus baccatus* a) *in vitro* establishment of shoot tips b) *in vitro* establishment of stem node sections c) multiplication stage d) rooting stage e) acclimatization

**RAPD analysis:** RAPD analysis was performed for screening six micropropagated plants which were multiplied with different concentrations of hormone (1, 7, 8, 12, 13 and 23 in Table 4), produced plant *in vitro* and their mother plant to detect the effect of hormone on the genetic stability. The amplification profile of the 8 primers produced 29 polymorphic bands out of 73 reproducible products Table-6. This corresponds to 39.72 % polymorphism level. The size of amplified products varied from 190 bp to < 2500 bp. All the primers were found to be polymorphic and produced different percentages of polymorphism. The number of amplicons per primer ranged from 7 (OPA-17 and OPA-11) to 14 (OPA-13), while the number of polymorphic bands per primer ranged from 1 (OPA-11 and OPZ-13) to 9 (OPB-18). Similar findings Delaia *et al*. (2000) were also reported total of 62 amplification products from 15 random 10-mer primers out of which 39 products were polymorphic in subcultures of clone A of *Eucalyptus*. Similarly Goto *et al*. (1998) obtained total of 134 scorable bands using 30 decamer primers in micropropagated shoots of *Pinus thunburghii* and the number of bands for each primer varied from 2 to 7.

Among the various methods developed to micropropagate plants, enhanced axillary branching is adventageaus because it is simple and the propagation rate is relatively high (Pierik, 1991). Micropropagation through axillary buds / organized meristems is generally considered to be a low risk method for genetic in stability (Pierik, 1991), because the organized meristems are generally more resistant to genetic changes as compared to unorganized callus under *in vitro* conditions (Shenoy and Vasil, 1992). Long duration of cultures of *Ochradenus baccatus* (12 subcultures) may be one of the factors causing variations. This is in agreement with Modgil *et al*. (2005) who found that long duration of cultures of apple MM106 might be one of the factors causing variations.

From the present study it is clear that somaclonal DNA sequence variation is present in micropropagated plants of *Ochradenus baccatus*. The polymorphism in amplified bands might result from changes in either the sequence of the primer binding site (e.g. point mutations) or change which alter the size or prevent successful amplification of target DNA (e.g. insertions, deletions, inversions) (Williams *et al*., 1993). The number of copies of particular sequences in the genome may vary after cell culture resulting in deamplification and amplification of genes (Larkin *et al*., 1989). Rani *et al*. (1995) reported variation to the extent of 26% in micropropagated plants of *populus deltoids*. Parani *et al*. (1997) found 56% polymorphism in 20 micropropagated plants of *piper longum*. It can be inferred from this study that some of the shoots become unstable under *in vitro* conditions and / or more prone to *in vitro* induced stress leading to somaclonal variation. Complete homology between parents and micropropagated plants was lacking. Our results suggest that RAPD technique can be successfully used to assess genetic variations in micro-propagated plants Fig-2. It also demonstrates that genetic integrity of micropropagated plants should invariably be confirmed before transfer of hardened plants to field.

**Table-6:** Description of eight decamer primers used for fingerprint analysis of micropropagated plants of *Ochradenus baccatus*.

| Primer | Total no. amplicons | No. polymorphic amplicon | % of polymorphic amplicon | Size range (bp) |
|---|---|---|---|---|
| OPA-02 | 8 | 3 | 37.5 | 1600-500 |
| OPA-17 | 7 | 2 | 28.5 | 1500-350 |
| OPA-13 | 14 | 4 | 28.5 | 1500-200 |
| OPB-13 | 10 | 6 | 60 | 2500-400 |
| OPB-18 | 10 | 9 | 90 | 1500-190 |
| OPA-11 | 7 | 1 | 14.2 | 1353-350 |
| OPZ-12 | 9 | 3 | 33.3 | 1353-410 |
| OPZ-13 | 8 | 1 | 12.5 | 1313-340 |
| | 73 | 29 | 39.7 | |

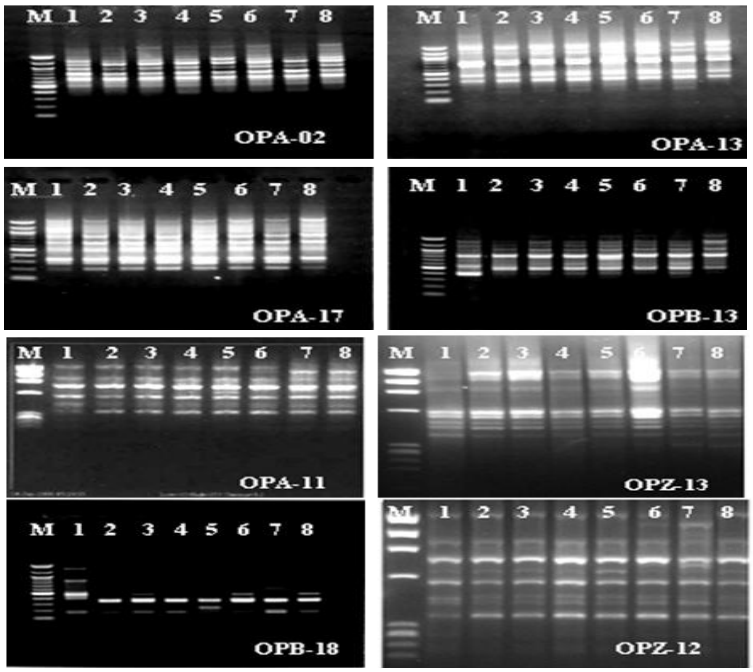


**Figure-2:** RAPD profiles of micropropagated plants of *Ochradenus baccatus*. Lane M: 100bp ladder (MBI Fermentas).Lane 1 represents the mother plant, lanes 2, 3, 4, 5, 6 and 7 represent the micropropagated plants under different concentrations of hormones and lane 8 represents the acclimatized plant.

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