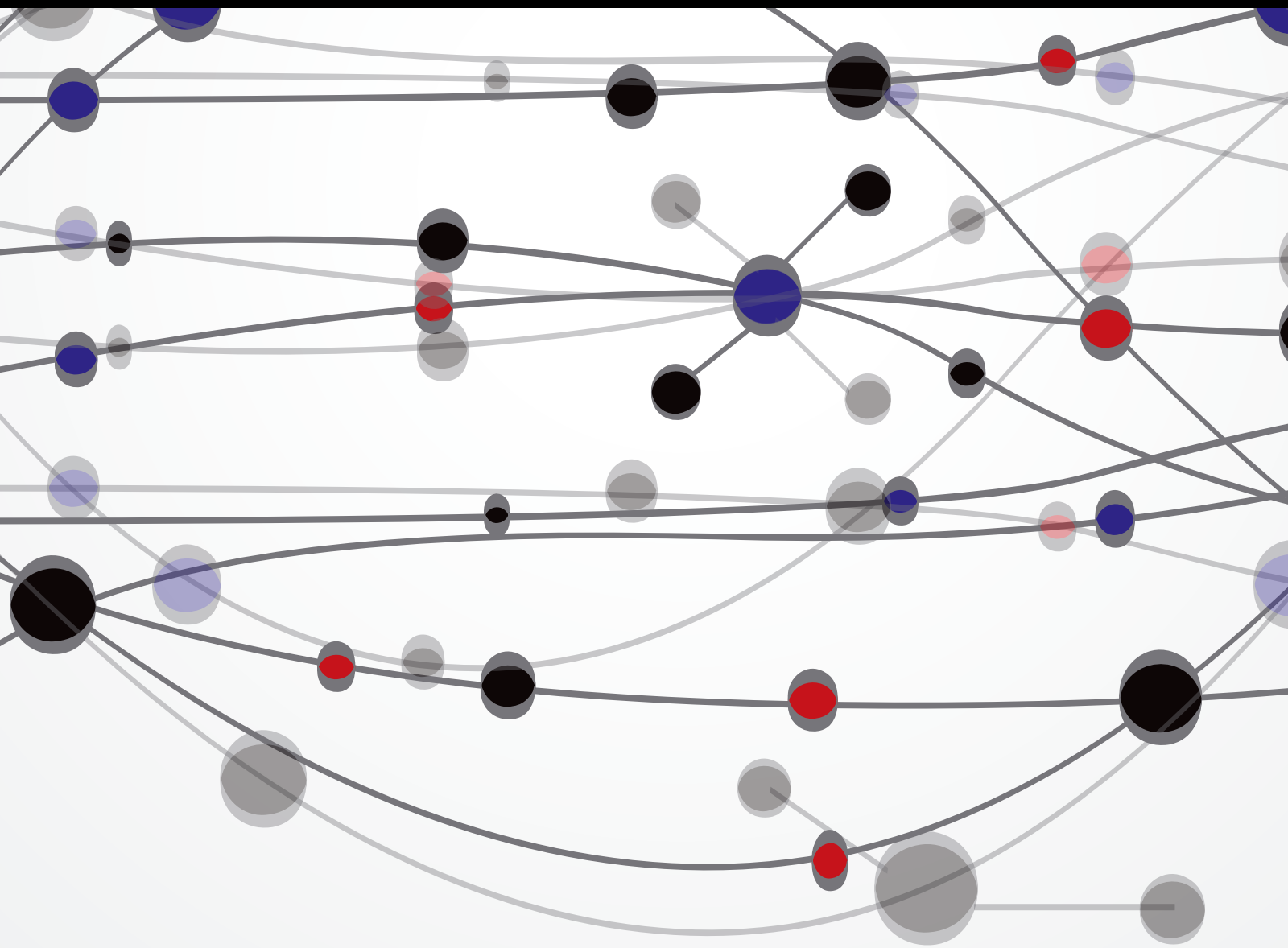
The Scientific World Journal

Plant Biotechnology

Guest Editors: Khalid Mahmood Khawar, Selma Onarici, Cigdem Alev Ozel, Muhammad Aasim, Allah Bakhsh, and Abdul Qayyum Rao



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Contents

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Editorial
Plant Biotechnology

Khalid Mahmood Khawar,[1] Selma Onarici,[2] Cigdem Alev Ozel,[3] Muhammad Aasim,[4] Allah Bakhsh,[5] and Abdul Qayyum Rao[6]

[1] *Department of Field Crops, Ankara University, 06110 Ankara, Turkey*
[2] *The Scientific and Technical Council of Turkey Genetic Engineering and Biotechnology Institute, Gebze, 41470 Kocaeli, Turkey*
[3] *Department of Biology Education, Gazi University, 06500 Ankara, Turkey*
[4] *Department of Biology, Karamanoglu Mehmetbey University, 70200 Karaman, Turkey*
[5] *Department of Agricultural Sciences, Section Plant Molecular Virology, University of Bologna, 40127 Bologna, Italy*
[6] *Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, 87 West Canal Bank Road, Thokar Niaz Baig, Lahore 53700, Pakistan*

Correspondence should be addressed to Khalid Mahmood Khawar; kmkhawar@gmail.com

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It is a privilege, pleasure, and honour to present this special issue to the international scientific community. The editorial board is confident that it is a truly good publication; as the papers published in this issue cover a broad spectrum of topics on plant biotechnology, that could be of wide interest.

This special issue received 20 papers, of which 2 papers were withdrawn and 4 were rejected (not for their quality but for their relatedness to the special issue).

The guest editorial board of this issue would like to mention that the published papers in this special issue contain one paper on molecular and genetic diversity from China that studied 115 sugarcane genotypes based on five genomic simple sequence repeat marker (gSSR) loci and 88 polymorphic alleles of loci as detected by capillary electrophoresis. The results indicated large intrapopulation genetic variation compared with interpopulation variation. The knowledge obtained in this study should be useful for future breeding programs.

One paper discusses the problem of mantled fruits as a result of somaclonal variation in oil palm plantlets regenerated via tissue culture. The molecular aspects of the occurrence possibly due to gene repression such as DNA methylation, histone methylation, and histone deacetylation are discussed. This paper described the total protein polymorphism profiles of somaclonal variants of oil palm and the effects of histone deacetylation on this phenomenon.

One paper presents cytogenetic study of Papaver species that introduced a new concept by developing ice cold water instead of bromonaphthalene for chromosome studies.

The papers on plant cell and tissue culture include a review on genetic transformation in citrus; micropropagation of *Origanum acutidens* (Hand.-Mazz.) Ietswaart using stem node explants; use of tissue culture techniques for producing virus-free plant in garlic; adventitious shoot regeneration from leaf explant of aquatic plant *Hygrophila polysperma (Roxb.) T. Anderson)*; secondary somatic embryogenesis in *Hovenia dulcis* Thunb.; effects of IAA, IBA, NAA, and GA3 on rooting and morphological features of *Melissa officinalis* L. stem cuttings; comparison of different methods for separation of haploid embryo induced through irradiated pollen and their economic analysis in melon (*Cucumis melo* var. inodorus); and comparative studies on cellular behaviour of carnation (*Dianthus caryophyllus* Linn. Cv. Grenadin) grown *in vivo* and *in vitro* for early detection of somaclonal variations and the influence of 1-triacontanol on the growth, flowering, and quality of potted *Bougainvillea glabra* var. "Elizabeth Angus" under natural conditions. Still in another paper published in this issue, there is important information about "*Annotation of differentially expressed genes in the somatic embryogenesis of Musa and their location in the banana genome*." The important information about TDFs

sequences opens new possibilities for an in-depth study on molecular and biochemical research pertaining to zygotic and somatic embryogenesis in *Musa* species.

These highly esteemed papers are easily available in the form of open access papers at the website of the journal (http://www.hindawi.com/journals/tswj/psi/biotechnology/). The journal is indexed in many important international databases including Science Citation Index Expanded, Zoological Record, and BIOSIS Previews.

Acknowledgments

The editors thank the authors and the management of the journal for their continued interest, that helped the guest editors to maintain the highest ethical standards to ensure publication of high-quality nonplagiarised original scientific material in the journal. It has all been possible due to the rigorous labour, hard work, dedication, and team work at each end.

Khalid Mahmood Khawar
Selma Onarici
Muhammad Aasim
Abdul Qayyum Rao
Cigdem Alev Ozel
Allah Bakhsh

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Research Article

A Comparison of Ice Cold Water Pretreatment and α-Bromonaphthalene Cytogenetic Method for Identification of *Papaver* Species

Amir Rezaei Osalou,[1] Sheida Daneshvar Rouyandezagh,[2] Behrouz Alizadeh,[1] Celal Er,[1] and Cafer Sirri Sevimay[1]

[1] *Department of Field Crops, Faculty of Agriculture, Ankara University, 06110 Diskapi, Turkey*
[2] *Department of Agricultural Biotechnology, Faculty of Agriculture, Namik Kemal University, 59030 Tekirdag, Turkey*

Correspondence should be addressed to Sheida Daneshvar Rouyandezagh; sdaneshvar@nku.edu.tr

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The plants belonging to many species in genus Papaver are very similar and have very small chromosomes that make identification very difficult. The study aimed to compare the effects of α-bromonaphtalene and ice cold water pretreatment to identify chromosomes of Papaver species collected from different areas of Iranian West Azerbaijan and Turkish Van, Agri and, Hakkari provinces. The seeds were germinated in Jacobson trays at 24°C under continuous light. Thereafter, roots from 1.5 cm long plantlets were pretreated with α bromonaphtalene for 15, 30, and 45 min or at 0°C in ice cold water for 24 h before fixing, hydrolyzation, and feulgen staining. The ice cold water pretreatment was more appropriate and easy to determine chromosomes. Seeds from seven samples did not germinate. Sixty samples out of the rest of 62 samples were identified as *P. pseudo orientale*, one sample was identified as *P. bracteatum*, and another as *P. orientale*. This is the first study that used ice cold water to determine the chromosomes in papaver species. It is hoped that it will also facilitate to determine chromosome number and identify other papver species.

1. Introduction

Papaver pseudo-orientale ($2n = 6x = 42$), *P. bracteatum* ($2n = 2x = 14$), and *P. orientale* ($2n = 4x = 28$) are three important species belonging to the section Oxytona, which resemble very closely. These species were first brought to Europe in the early eighteenth century by Tournefort and were introduced as "oriental poppy" [1].

P. pseudo-orientale grows at an altitude of 1600–2200 m at moist places in Iran and Turkey [1], which is used as an ornamental plant because of its beautiful and attractive flowers. *P. pseudo-orientale* displays erect flower buds with orange-red petals with or without basal marks. It is rich in isothebaine [2], macranthaline, orientalidine, and salutaridine, which makes it an important medicinal plant [3] of pharmaceutical importance. Concentration of these alkaloids varies in capsules, leaves, stems, and roots. The alkaloid spectrum and content also differ during plant growth and development. Thebaine is the most important alkaloid obtained from this group of plants.

Identification of the plant species is difficult, which is generally done after consultating taxonomic keys to be sure that specimen is identified correctly. However, phenotypic appearance of the three species *P. pseudo-orientale* ($2n = 6x = 42$), *P. bracteatum* ($2n = 2x = 14$) and *P. orientale* ($2n = 4x = 28$) is very similar and there is every likelihood that plants are identified erroneously. Therefore, another important way to determine the plant species is to determine the chromosome numbers, which are a concrete feature to remove any ambiguity among the three species of section Oxytona [1, 4]. The ploidy level provides clear delimitation of species in this section. According to a systematic study by Goldblatt [1], the hexaploid *P. pseudo-orientale* ($2n = 42$) is regarded as transient form between (or an allopolyploid hybrid of) the diploid *P. bracteatum* ($2n = 14$) and the tetraploid *P. orientale* ($2n = 28$).

Cytogenetic analyses reveal formation of multivalents at diakinesis, in the polyploid species like *P. pseudo-orientale* and in its hybrids with diploid species. This finding demonstrates the autoploid nature of *P. pseudo-orientale* and proves that *P. bracteatum* is as its ancestor. Similarly, the similarities found in isozyme variation and in chloroplast DNA restriction patterns between and within the three species of section Oxytona also strongly indicate the autoploid nature of this section [5, 6].

Producing good somatic metaphase spreads is hindered by combined difficulties of obtaining large number of dividing cells and of spreading and staining the chromosomes well. The thickness and length of chromosomes are other important factors that affect visibility of chromosomes during metaphase. It is also important to catch the best view at appropriate stage before fixation of chromosomes. Over the years, many methods have been developed for preparing somatic chromosomes, and they are generally made up of four stages: first, the seed is germinated and the actively growing root tip is collected; second, a pretreatment using ice cold water for a definite period of time or 8-Hydroxyquinoline, Colchicine, Paradichlorobenzene, or α-monobromonaphthalene for a definite period of time prior to fixation to inhibit spindle formation, preventing the congression of chromosomes to the metaphase plate; third is fixation in any of a large range of solutions; fourth, preparation of the root tips by hydrolysis, followed by staining.

Most studies pertaining to chromosome counts in *P. pseudo-orientale* make use of α-monobromonaphthalene as pretreatment. This study aimed to compare the effects of α-monobromonaphthalene and 0°C water pretreatment for 24 hours on chromosome visibility of Papaver species, which is an important method for observing cereal chromosomes, where this pretreatment is preferred over other methods when chromosomes of a large number of plants are to be studied.

2. Materials and Methods

2.1. Plant Material. The study included 69 different populations of Papaver section Oxytona collected from Van, Hakkari and Agri provinces of Eastern Anatolian Turkey and two samples of Iran origin, collected from West Azerbaijan Province of Iran. The seeds were germinated between moist sandwiched filter papers on Jacobson trays at 24°C under continuous light. Thereafter, when the plants increased to lengths of 1, 1.5, 2, and 3 cm, they were collected for preparation of preparates in the laboratory. At least 5 preparates were made from each sample unless specified.

2.2. α-Monobromonaphthalene Method. The roots of selected plants of sample number 414 were pretreated with α-monobromonaphthalene for 15, 30, and 45 min at room temperature followed by rinsing of pretreated roots with distilled water for 4-5 min.

2.3. Fixation. Thereafter, in order to fix or stop the chromosomes at the desired stage of cell division, the roots were treated with Carnoy's solution consisting of 1 part glacial acetic acid and 3 parts ethanol (95 to 100%). This fixative was prepared fresh each time. The material was kept in the fixative for 30 minutes at room temperature followed by rinsing with bidistilled water.

2.4. Hydrolysis. Then roots were hydrolysed for 8 minutes in 1 N HCl at 60°C followed by washing with bidistilled water.

2.5. Staining. Hydrolyzed specimens were stained with Feulgen for 30 minutes under darkness at room temperature followed by washing with bi-distilled water before making preparates.

2.6. Making Preparates. The roots were quickly transferred into 45% acetic acid dropped on a microscopic slide previously. Then 2-3 mm-long meristematic (well stained) sides of roots tips were cut discarding remaining portions of the roots. The well-stained root tips were carefully sliced using a sharp blade (Gillette) dropping 45% acetic acid little by little to avoid the escaping of sliced tissues from the blade and preventing drying of the acetic acid. These tissues were mixed up to get a homogenous liquid using a needle and closed slowly beginning from one side to another side by a coverslip. Thereafter, the surplus liquid was absorbed using blotting paper. To avoid the slipping of coverslip over the slide, it was kept firmly held using thumb and was carefully knocked using bottom of a pencil to flatten the cells, avoiding chromosome diffusion and facilitating easy observation of chromosomes without overlapping. Air bubbles created between glass slide and coverslip were eliminated by dropping 45% acetic acid at the edge of each coverslip. The extra-acetic acid was absorbed using blotting paper. The preparates were observed using a Nikon microscope to count and distinguish chromosomes.

2.7. Pretreatment Using Melting Ice (Melting Ice Method). Germination and selection of the plants was done as suggested previously. However, the roots were pretreated by placing the selected plants at 0°C in the melting ice, inside a refrigerator at 4°C without cutting them for 24 hours.

2.8. Fixation. Thereafter, the roots of the plants were cut and treated with Carnoy's solution 1 consisting of ethanol-acetic acid (3 : 1) for 30 min at room temperature followed by washing with distilled water.

It was followed by hydrolysis for 8 min in 1 N HCl, at 60°C, staining with Feulgen for 30 min at room temperature, washing with bidistilled water, and preparation of slides, as described above.

3. Results and Discussion

3.1. Using α-Monobromonaphthalene Method for Chromosome Determination. A good experimental procedure has to show clear and sharp chromosomes that were not possible when α-monobromonaphthalene method was used. The analysis of root tip slides prepared from 1, 1.5, 2, and 3 cm long plants

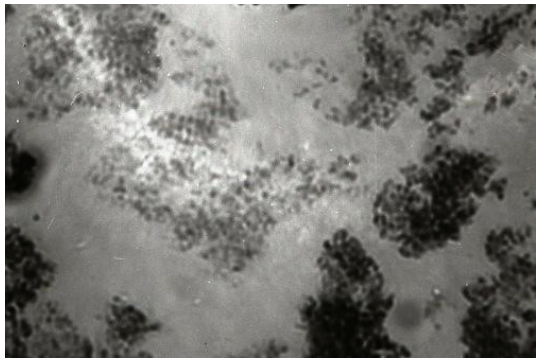

Figure 1: $2n = 6x = 42$ chromosomes belonging to sample 504 K of *P. pseudo orientale* collected from Dibekli koprusu, Van Turkey pretreated with α-monobromonaphthalene for 45 minutes.

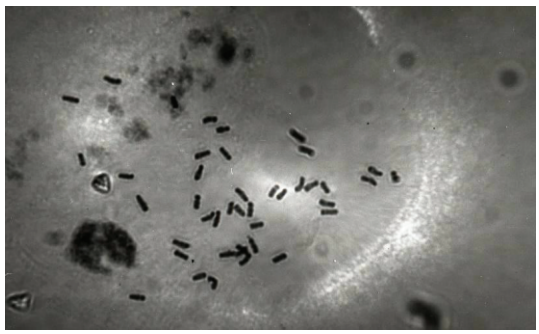

Figure 2: (10×40), $2n = 6x = 42$ chromosomes belonging to sample 419 K of *P. pseudo orientale* collected from Dogubayezit-Igdir Road and Agri province, Turkey, pretreated with 0°C melting ice for 24 hours.

of *P. pseudo-orientale* using sample number 504 K collected from Yuksekova Dibekli koprusu, Turkey, showed that the species exhibited lower and blurred expressions (Figure 1). The chromosomes were difficult to count, unrecognizable, and dim. Moreover, the treatment resulted in more diffusion of chromosomes. Using α-monobromonaphthalene method for chromosome determination was not helpful. Martens and Reisch [7] observed that the influence of the time of sampling in the activity of cell mitosis is very important. It is thought using α-monobromonaphthalene method for chromosome analysis may have negatively affected the roots tips during pretreatment with monobromonaphthalene causing some internal rearrangements in the root tips that caused difficulty in observing and counting the chromosomes at the time of mitotic division of root cells. It also decreased the quality of the studied chromosome. It seemed as if α-bromonaphthalene method was incompetent and encountered problems in chromosome counting.

3.2. Using Ice Cold Water Method for Chromosome Determination. Optimization of method for chromosome counts was made using sample number 419 K collected from Dogubayezit-Igdir Road location, and using ice cold water method for chromosome determination was very helpful. The preprates were initially pretreated in ice cold water.

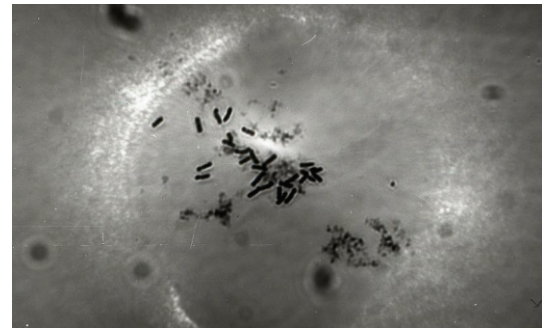

Figure 3: (10×40), $2n = 6x = 42$ chromosomes belonging to sample 419 K of 446 *P. pseudo orientale* collected from Caldıran, Gulhisar, and Van provinces, Turkey, pretreated with 0°C melting ice for 24 hours.

The chromosomes were shorter and thicker. The results also showed better staining and separation of chromosomes, so that they were visible and countable. This elevated the opportunity of observing and counting the chromosomes easily. Irrespective of seedling length, the method could easily help in helping the chromosome counts. The chromosomes were recognizable and clear and had high expression. Moreover, they were not difficult to count under the microscope, yet the initial experiments showed that out of the plants that increased to a length of 1, 1.5, 2, and 3 cm, 1.5 cm long plants were the best suited for chromosome determination (Figure 2). This showed that the plant material assayed in this study to determine the chromosome numbers *Papaver* species was most active at this stage of growth showing active mitotic division for easy chromosome count. The roots tips were hydrolyzed by incubation in 1 N HCl at 60°C for 8 min followed by rinsing in distilled water for 2-3 min. to catch metaphase. This time (8 min) in hydrolyzing procedure is necessary for elimination of membrane cytoplasm. Hydrolyzed specimens were best stained with Feulgen for 30 minutes followed by washing with bi-distilled water before making preparates. Acetic acid was used to dissolve the fatty acid and waxy secretions, facilitating the penetration of the fixative. The observed chromosomes number is in agreement with previous studies showing the chromosome number of 42 in *P. pseudo-orientale*. Once the method was optimized, it was used to identify the rest of 68 samples.

Therefore subsequent experiments used 1.5 cm long plants for determination of chromosomes and identification of species.

A total of 69 samples were investigated. Their sample codes, subregion/region from where they were collected, period of germination (days), Germination (%), and species that were identified are given in Table 1.

Martens and Reisch [7] observed that the influence of the time of sampling in the activity of cell mitosis is very important. It is thought using ice cold water method for chromosome determination may have positively affected the roots tips during pretreatment ice cold water that helped in easy determination of chromosome counts at the time of

Table 1: Sample code, sub region/region, number of days to germinate seeds, germination percentage, and the identified Papaver species from the samples collected from various regions of Turkey and Iran.

Number	Sample code	Sub-region/region	Period of germination (day)	Germination (%)	Species
1	414	Kayak T./Agrı	8	30	*P. Pseudo-orientale*
2	415	Kayak T./Agrı	8	30	*P. Pseudo-orientale*
3	416	Kayak T./Agrı	8	10	*P. Pseudo-orientale*
4	417	Kayak T./Agrı	7	90	*P. Pseudo-orientale*
5	419 K	Igdır yolu/Agrı	7	90	*P. Pseudo-orientale*
6	421	Hamur-Tutak/Agrı	6	60	*P. Pseudo-orientale*
7	423	Hamur-Tutak/Agrı	6	80	*P. Pseudo-orientale*
8	424	Hamur-Tutak/Agrı	10	60	*P. Pseudo-orientale*
9	425	Hamur-Tutak/Agrı	10	2.38	*P. Pseudo-orientale*
10	426	Hamur-Tutak/Agrı	—	0	Nil-
11	427	Hamur-Tutak/Agrı	8	40	*P. Pseudo-orientale*
12	428	Hamur-Tutak/Agrı	8	30	*P. Pseudo-orientale*
13	429	Hamur-Tutak/Agrı	11	10	*P. Pseudo-orientale*
14	430	Hamur-Tutak/Agrı	11	10	*P. Pseudo-orientale*
15	431	Hamur-Tutak/Agrı	11	10	*P. Pseudo-orientale*
16	432	Hamur-Tutak/Agrı	8	15	*P. Pseudo-orientale*
17	434	Hamur-Tutak/Agrı	7	70	*P. Pseudo-orientale*
18	435	Hamur-Tutak/Agrı	—	0	Nil-
19	437	Hamur-Tutak/Agrı	7	50	*P. Pseudo-orientale*
20	440	Hamur-Tutak/Agrı	9	70	*P. Pseudo-orientale*
21	441	Hamur-Tutak/Agrı	7	30	*P. Pseudo-orientale*
22	442	Hamur-Tutak/Agrı	—	0	Nil-
23	444 K	Safak cesmesi/Agrı	6	40	*P. Pseudo-orientale*
24	445	Caldıran So guksu/VAN	8	80	*P. Pseudo-orientale*
25	446	Caldıran Gulhisar/VAN	10	25	*P. Orientale*
26	448	Bahcesaray/VAN	8	60	*P. Pseudo-orientale*
27	449	Bahcesaray/VAN	8	70	*P. Pseudo-orientale*
28	450	Bahcesaray/VAN	6	90	*P. Pseudo-orientale*
29	451	Bahcesaray/VAN	7	2.38	*P. Pseudo-orientale*
30	452	Bahcesaray/VAN	7	90	*P. Pseudo-orientale*
31	453	Bahcesaray/VAN	8	90	*P. Pseudo-orientale*
32	454	Bahcesaray/VAN	8	90	*P. Pseudo-orientale*
33	455	Bahcesaray/VAN	8	90	*P. Pseudo-orientale*
34	456	Bahcesaray/VAN	6	80	*P. Pseudo-orientale*
35	457	Bahcesaray/VAN	7	90	*P. Pseudo-orientale*
36	458	Bahcesaray/VAN	7	90	*P. Pseudo-orientale*
37	459	Bahcesaray/VAN	8	90	*P. Pseudo-orientale*
38	462 K	Baskale Guzeldere/VAN	7	90	*P. Pseudo-orientale*
39	463	27 km to Baskale/VAN	7	80	*P. Pseudo-orientale*
40	465	Alan vadisi-Cesme yanı/HAKKARI	8	90	*P. Pseudo-orientale*
41	466	Alan vadisi-Cesme yanı/HAKKARI	8	70	*P. Pseudo-orientale*
42	468	Alan vadisi/HAKKARI	7	90	*P. Pseudo-orientale*
43	469	Alan vadisi/HAKKARI	8	70	*P. Pseudo-orıentale*

Table 1: Continued.

Number	Sample code	Sub-region/region	Period of germination (day)	Germination (%)	Species
44	470 K	Alan vadisi/HAKKARI	7	70	*P. Pseudo-orientale*
45	472	Alan vadisi Karakol/HAKKARI	8	50	*P. Pseudo-orientale*
46	473	Alan vadisi Karakol/HAKKARI	8	60	*P. Pseudo-orientale*
47	475	Alan vadisi Karakol/HAKKARI	9	Very Low	*P. Pseudo-orientale*
48	476	Alan vadisi Karakol/HAKKARI	9	Very Low	*P. Pseudo-orientale*
49	477	Alan vadisi Karakol/HAKKARI	8	90	*P. Pseudo-orientale*
50	478	Alan vadisi Karakol/HAKKARI	—	0	Nil-
51	479	Alan vadisi Karakol/HAKKARI	10	90	*P. Pseudo-orientale*
52	480	Alan vadisi Karakol/HAKKARI	7	80	*P. Pseudo-orientale*
53	481 K	Alan vadisi Karakol/HAKKARI	7	90	*P. Pseudo-orientale*
54	486	Semdinli/HAKKARI	10	15	*P. Pseudo-orientale*
55	490	Yuksekova Semdinli/HAKKARI	8	50	*P. Pseudo-orientale*
56	491	Yuksekova Semdinli/HAKKARI	8	Very Low	*P. Pseudo-orientale*
57	492	Yuksekova Semdinli/HAKKARI	8	40	*P. Pseudo-orientale*
58	493	Yuksekova Semdinli/HAKKARI	—	0	Nil-
59	494	Yuksekova Semdinli/HAKKARI	7	70	*P. Pseudo-orientale*
60	495	Yuksekova Semdinli/HAKKARI	—	0	Nil-
61	496	Yuksekova Semdinli/HAKKARI	12	20	*P. Pseudo-orientale*
62	497	Yuksekova Semdinli/HAKKARI	13	10	*P. Pseudo-orientale*
63	500	Yuksekova Copluk/VAN	6	80	*P. Pseudo-orientale*
64	501	Yuksekova Copluk/VAN	8	70	*P. Pseudo-orientale*
65	503 K	Yuksekova kopruyanı/VAN	8	70	*P. Pseudo-orientale*
66	504 K	Dibekli Koprusu/VAN	8	90	*P. Pseudo-orientale*
67	7	Qushju Iranian province of West Azerbaijan, Iran	8	70	*P. bracteatum*
68	F1 (P.b × P.o)	Ankara University, Turkey	—	0	Nil
69	9	Qushju Iranian province of West Azerbaijan, Iran	9	90	*P. Pseudo-orientale*

mitotic division of root cells. It also increased the quality of the chromosome studies.

A review of the Table 1 shows that out of 69 samples, the seeds that belonged to seven samples (coded as 426, 435, 442, 478, 493, 495 and F1 (P.b × P.o) did not germinate and their chromosomes could not be counted (Table 1). Rest of the 62 samples except sample number 25 coded as 446 collected from Caldıran Gulhisar/VAN locations and sample number 67 coded as 7 collected from Iranian province of West Azerbaijan had chromosome number of 28 (Figure 3) and 14 and were identified as as *P. bracteatum* and *P. orientale*, respectively.

The *P. pseudo-orientale* seeds collected from different locations took 6–13 days to germinate. Whereas, the seeds of *P. bracteatum* took 8 days, *P. orientale* seeds took 10 days to germinate. Germination percentage of *P. pseudo-orientale* seeds was 2.38–90%, the seeds of *P. bracteatum* had seed germination percentage of 70%, and *P. orientale* had germination percentage of 25%.

Results of this chromosomal study have proven useful in plant taxonomy and phylogenetic analysis. In this sense, our results are helpful in identifying and separating *P. pseudo-orientale* species collected from Hakkari, Van, and Agri, and they noted no differences among them in terms of chromosome numbers. The results are in agreement with morphological features of the plants collected from these locations and previous studies by Goldblatt [1].

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Research Article

Induction and Analysis of the Alkaloid Mitragynine Content of a *Mitragyna speciosa* Suspension Culture System upon Elicitation and Precursor Feeding

Nor Nahazima Mohamad Zuldin,[1] Ikram Md. Said,[2,3] Normah Mohd Noor,[3] Zamri Zainal,[1,3] Chew Jin Kiat,[1,3] and Ismanizan Ismail[1,3]

[1] *School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*
[2] *School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia*
[3] *Institute of System Biology, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia*

Correspondence should be addressed to Ismanizan Ismail; maniz@ukm.my

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This study aimed to determine the effects of different concentrations and combinations of the phytohormones 2,4-dichlorophenoxy acetic acid (2,4-D), kinetin, 6-benzylaminopurine (BAP), and 1-naphthaleneacetic acid (NAA) on callus induction and to demonstrate the role of elicitors and exogenous precursors on the production of mitragynine in a *Mitragyna speciosa* suspension culture. The best callus induction was achieved from petiole explants cultured on WPM that was supplemented with 4 mg L^{-1} 2, 4-D (70.83%). Calli were transferred to liquid media and agitated on rotary shakers to establish *Mitragyna speciosa* cell suspension cultures. The optimum settled cell volume was achieved in the presence of WPM that contained 3 mg L^{-1} 2,4-D and 3% sucrose (9.47±0.4667 mL). The treatment of cultures with different concentrations of yeast extract and salicylic acid for different inoculation periods revealed that the highest mitragynine content as determined by HPLC was achieved from the culture treated with 250 mg L^{-1} yeast extract (9.275 ± 0.082 mg L^{-1}) that was harvested on day 6 of culturing; salicylic acid showed low mitragynine content in all concentrations used. Tryptophan and loganin were used as exogenous precursors; the highest level of mitragynine production was achieved in cultures treated with 3 μM tryptophan and harvested at 6 days (13.226 ± 1.98 mg L^{-1}).

1. Introduction

Mitragyna speciosa is a medicinal tree in the Rubiaceae family that is native to Southeast Asia. It has been traditionally used in Thailand and Malaysia for its psychoactive properties; however, its use in these countries is now illegal. In Southeast Asia, the fresh leaves are commonly chewed, often continuously, by workers or manual laborers who seek its numbing, stimulatory effect. The leaves and bark of *M. speciosa* are used to cure opium addiction. These tissues contain many alkaloids, including mitragynine, mitraphylline, and 7-hydroxymitragynine; the latter is currently thought to be the most likely candidate for the primary active chemical in the plant. Mitragynine is the dominant alkaloid, and it has been assumed to be the physiologically active constituent that has morphine-like properties. It confers pain-threshold elevating and antitussive properties but lacks addictive properties [1].

Currently, the natural habitats of many plants are disappearing due to environmental and geopolitical instabilities; this loss of natural habitat for plants makes it difficult for human to acquire important secondary metabolites and prevents the discovery of many potentially useful compounds [2]. Important plant-derived drugs can still be obtained commercially by extracting the compounds from their whole-plant sources. The chemical synthesis of these compounds often results in the loss of their activity. The compounds

contain highly complex structures with many chiral centers, and this complexity may contribute to their biological activities and to the difficulty in their economical synthesis [3]. Therefore, it is important to use a new alternative method to improve the content and productivity of the active ingredients in these plants. Plant tissue culture methods have been developed for many other endangered medicinal plants, such as *Curculigo orchioides* [4], *Podophyllum hexandrum* [5], *Hypoxis hemerocallidea* [6], and *Saussurea involucrata* [7]. Plant cell culture is considered to be a promising alternative for producing bioactive compounds that are difficult to be obtained by chemical synthesis or plant extraction [3]. Tissue culture is an attractive method because each plant cell culture exhibits totipotency, wherein the cell mass contains the full set of genes that are necessary for all of the plant functions, including secondary metabolism [8]. Cell culture systems are useful for the large-scale culture of plant cells, which produces a continuous and reliable source of secondary metabolites that can be purified easily due to the absence of significant levels of pigments. This approach avoids all seasonal plant growth constraints and eliminates geographic barriers for the production of secondary metabolites [9].

The major limitation in the production of secondary metabolites by plant cell culture technology is the low yield of secondary metabolites. The yield could be improved by standardizing the culture environment [10] and manipulating plant cell cultures to improve the production of target compounds by employing elicitors, abiotic stresses, and other approaches regardless of their mechanism [11]. The synthesis of target secondary metabolites in plant cell tissue cultures can be induced by applying physical, chemical, and biological elicitors. These elicitors mimic the effects of stresses and thereby activate the plant biochemical system; this induction results in the increased production of secondary metabolites in plant tissues [12]. The elevated production of desired products by elicitation has been reported in many studies, such as the production of indole alkaloid ajmalicinine from *Catharanthus roseus* cultures that were elicited by the fungi *Trichoderma viride* [13], rosmarinic acid and eugenol from *Ocimum basilicum* that was elicited by chitosan [14], beta-amyrin from *Medicago truncatula* that was elicited by a yeast elicitor [15], and taxol from *Taxus chinensis* [16] and ginsenoside from *Panax* ginseng that were elicited by methyl jasmonate [17]. It also has been reported that the addition of precursors or intermediate compounds involved at the beginning of the secondary metabolic biosynthetic pathway to the culture media sometimes stimulates the production of secondary metabolites [18]. Several attempts to induce or increase the production of plant secondary metabolites by supplying precursors or intermediate compounds have been performed, such as in the production of the alkaloid lunarine from *Lunaria annua* that was treated with phenylalanine [19], ajmalicine and strictosidine from *Catharanthus roseus* that was treated with secologanin, loganin, or loganic acid [20] and tryptophan [13], vanillin and capsaicin from *Capsicum frutescence* that was treated with the ferulic acid anvanyllyamine [21], anthocyanin from strawberry cultures that were treated with phenylalanine [22], and bilobalide and ginkgolides from *Ginkgo biloba* that was treated with terpenoid [23]. Therefore, the major objectives of this study were to establish an *M. speciosa* tissue culture system and to manipulate the culture environment and cell culture by elicitation and precursor feeding to increase the production of the alkaloid mitragynine.

2. Materials and Methods

2.1. Chemicals. Mitragynine standards were obtained from the School of Chemistry Sciences and Food Technology, Faculty of Science and Technology, UKM; WPM medium was obtained from Duchefa; α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzylaminopurine (BAP), and kinetin were purchased from Sigma; yeast extract, salicylic acid, and tryptophan were purchased from Merck; and loganin was purchased from ChromaDex USA.

2.2. Plant Materials. *Mitragyna speciosa* plants were collected from Padang Siding, Perlis, and were grown at glass-house Biotechnology Laboratory, UKM. The petioles and young leaves were used as the explants in this experiment. The explants were washed thoroughly with running tap water for 30 min. The explants were then sterilized with 70% alcohol for 15 seconds and were washed three times. The explants were soaked for 30 min in 40% clorox bleach that contained three drops of Tween-20 and were then washed three times with distilled water. Next, the explants were dried on petri plates that contained a layer of filter paper. After trimming the cut size, the surface-sterilized explants were planted on the culture medium.

2.3. Callus Culture. In this study, woody plant medium (WPM) was supplemented with 3% sucrose as a carbon source. The WPM pH was then adjusted to pH 5.8 ± 0.1 with either 1 N HCl or KOH, and it was solidified with 0.7% agar before it was autoclaved at 121°C for 15 min. The different explants which is petiole and young leaves were cultured on various concentrations of 2,4-D (2, 4, 6, and 8 mg L^{-1}) and were tested to determine which explants produced an optimal callus induction. WPM that was supplemented with 2,4-D, NAA, BAP, and kinetin individually or in combination at different concentrations was also used to study the effects of the different media components on callus induction. The cultures were incubated in a growth chamber at 25 ± 2°C. Each experiment had 24 replicates of explants and was repeated three times. The percentage of callus induction per plate was documented for up to 8 weeks. The culture medium that was not supplemented with any plant growth regulators was used as the control in this study.

2.4. Establishment of Suspension Culture. The establishment of the suspension culture was initiated by inoculating 2 g of finely chopped callus into 25 mL of liquid WPM in a 100 mL Erlenmeyer flask; it was incubated on a rotary shaker at 125 rpm. The cell suspension cultures were then maintained at 25 ± 2°C. Different concentrations of 2, 4-D (1 to 5 mg L^{-1}) and sucrose (3 to 5% w/v) were tested to produce a rapid-growing and well-dispersed suspension culture of

Table 1: Effects of different concentrations of 2,4-D on the percentage of callus induction between leaf explants and petiole explants.

Concentration of 2,4-D (mgL^{-1})	Percentage of leaf explants that produced callus (%)	Percentage of petiole explants that produced callus (%)
0	4.167^{bc}	9.72^{d}
2	16.67^{ab}	36.11^{b}
4	20.83^{a}	70.83^{a}
6	4.167^{bc}	26.39^{bc}
8	0^{c}	19.44^{cd}
10	0^{c}	19.44^{cd}

Percentages within a column that have the same letter are not statistically significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Mitragyna speciosa. Each treatment contained three replicates. The growth of the *Mitragyna speciosa* cell suspension culture was measured by the settled cell volume (SCV). The SCV was determined by allowing the suspension culture to sediment for 5 min in a sterile, graduated tube.

2.5. Preparation of Elicitors and Precursors. Filter sterilized elicitors and precursors were added to the subcultures of *M. speciosa* suspension cultures at varying concentrations. Yeast extract and salicylic acid were used as the elicitors at final concentrations of 100, 250, and 500 mg L^{-1}, whereas tryptophan and loganin were used as the exogenous precursors at final concentration of 3, 6, and 9 μM. These compounds were added to the *M. speciosa* suspension culture to test mitragynine production. Suspension culture medium that lacked elicitors and precursors was used as the control in this experiment. The cells were harvested six days and twelve days after treated with elicitor and exogenous precursor to analyze the mitragynine content.

2.6. Extraction. To extract the alkaloids, the cells were separated from the liquid media and ground with liquid nitrogen. Approximately 8 g was collected before it was extracted with 50 mL of methanol for three days. The methanol extract was filtered, and new methanol was added to the remaining cells; this process was repeated three times. The methanol filtrates were combined and evaporated under reduced pressure. The crude methanol extract was dissolved in a 10% acetic acid solution; it was shaken well and allowed to stand overnight. The acidic filtrate was adjusted to pH 11 with sodium carbonate and extracted with chloroform. The chloroform extract then was washed, dried over anhydrous sodium sulfate, and evaporated to yield a dry, crude alkaloid extract.

2.7. HPLC Quantification of Mitragynine. The presence of mitragynine in the samples was verified by the comparison of the Rt and UV spectral peaks of the sample with the standard peak. The HPLC system consisted of a Waters 2707 autosampler, a Waters 600 controller, and a Waters 2998 photodiode array detector. The data were collected and processed using the Empower Software System.

The analytical method was performed using Column χBridge C18 (size 5 μm, 4.6 × 250 mm) from Waters. The mobile phase was a methanol-water mixture (80 : 20, v/v) and was filtered separately before it was mixed through a 0.22 μm nylon membrane filter. The flow rate was 0.6 mL/min, and the autosampler vials were at an ambient temperature of 25 ± 1°C. A sampler volume of 10 μL was injected, and the detector was set at 254 nm. A standard calibration curve was constructed by injecting different concentrations of mitragynine standard. The calibration curve for the mitragynine standard was linear (1 mg L^{-1}–5 mg L^{-1}) with a regression factor of 0.989240.

2.8. Statistical Analysis. Analysis of variance (ANOVA) was performed using the Statistical Analysis System (SAS) Version 9.0 software [24] to determine the significance of the treatment effects for each experiment. A P value of < 0.05 was considered to be significant.

3. Results

3.1. Plant Growth Regulator and Explants Effects on Callus Inductions. The petiole and leaf explants were placed on WPM that was supplemented with different concentrations of 2,4-D (2, 4, 6, 8, and 10 mg L^{-1}) for callus induction. The optimum callus induction was obtained from leaf explants that were WPM supplemented with 4 mg L^{-1} of 2,4-D which is 20.83% (Table 1). For petiole explants, the optimum callus induction percentage was obtained with WPM that was supplemented with 4 mg L^{-1} of 2,4-D (70.83%) (Table 1). This concentration significantly affected the callus induction when compared to the control and the other concentrations of 2,4-D. Leaf explants produced a low efficiency of callus induction in all of the treatments compared to petiole explants. The callus cultures derived from the leaf explants grew quite slowly in all of the tested concentrations. No callus induction was observed in leaf explants cultured on WPM that was supplemented with 8 or 10 mg L^{-1} of 2,4-D. The leaf explants that did not induce callus turned brown and died. In general, media that contain high auxin and low cytokinin concentrations promote cell proliferation that results in callus formation [25]. To study the effects of plant growth regulators (PGRs) on callus culture, different PGRs were tested at different concentrations and in various combinations on the petiole explants. The combination of 2,4-D (2, 4, and 6 mg L^{-1}) and kinetin (1 and 3 mg L^{-1}) did not provide a responsive effect on the callus induction percentage compared to WPM that was supplemented with 2,4-D alone (Table 2). However, the combination of BAP and NAA showed that the optimum callus induction percentage was only achieved by 50% of callus induction on WPM containing 0.5 mg L^{-1} BAP + 4 mg L^{-1} NAA (Table 3). The callus that was obtained from the petiole explants that were cultured with 2,4-D and kinetin was friable and whitish in color, while the callus that was obtained from BAP and NAA was more compact, hard, and globular in shape. To establish the cell suspension cultures, the callus from the petiole explant that was cultured on WPM that was supplemented with 4 mg L^{-1} 2,4-D was elected to initiate the *M. speciosa* suspension cultures.

Table 2: Effects of the combination 2,4-D and kinetin at different concentrations on the percentage of callus induction from petiole explants.

Treatments	Percentage of explants that produced callus (%) 2,4-D (mgL^{-1})			
	0	2	4	6
Kinetin (mgL^{-1})				
0	12.5^{d}	37.68^{b}	72.22^{a}	27.77^{bc}
1	26.387^{bc}	4.167^{d}	12.5^{d}	25^{c}
3	4.167^{d}	31.92^{bc}	36.11^{bc}	36.11^{bc}

Percentages within a column that have the same letter are not statistically significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Table 3: Effects of the combination NAA and BAP at different concentrations on the percentage of callus induction from petiole explants.

Treatments	Percentage of explants that produced callus (%) NAA (mgL^{-1})				
	0	2	4	6	8
BAP (mgL^{-1})					
0	6.943^{ef}	8.33^{def}	37.5^{b}	24.997^{c}	20.833^{cd}
0.5	4.167^{f}	19.443^{cde}	50^{a}	12.5^{def}	8.33^{def}

Percentages within a column that have the same letter are not statistically significantly different according to Duncan's multiple range test at $P \leq 0.05$.

3.2. Establishment of Cell Suspension Culture. To study the effects of auxin on the establishment of cell suspension culture, 2,4-D was used at concentrations from 1 to 5 mg L^{-1}. As shown in Figure 1, 3 mg L^{-1} of 2,4-D was found to induce the best growth among all of the treatments, and it produced the highest measurements of SCV (9.47 ± 0.4667 mL). As the day progressed, the percentage of cell viability for most of the treatments decreased, and the color of the callus darkened. This may have been caused by the secretion of phenolic compounds, which may have led to the death of the heterogeneous suspension cultures after 9 days of culture. Concentrations of 2,4-D that were higher than 3 mg L^{-1} did not show improvement of the cell growth. The growth pattern for the 2, 3, and 4 mg L^{-1} treatments showed an inactive sigmoidal shape. This may have been caused by the adaptation of the suspension cells to the newly inoculated environment during the lag phase; as they entered the log phase, the cells eventually adapted to the new environment and started to grow exponentially by utilizing the nutrients that were provided [26]. In the 1 mg L^{-1} 2,4-D treatment, the cells initially grew rapidly, but the growth subsequently decreased drastically after 6 days of culture; conversely, at 5 mg L^{-1} of 2,4-D, a slow growth pattern was initially observed, and the culture continued to increase at 12 days of culture, while the other treatments decreased after 9 days of culture (Figure 1).

In another experiment, the growth of the cell suspension culture was tested in the presence of 3 different concentrations of sucrose as a carbon source. Figure 2 showed callus

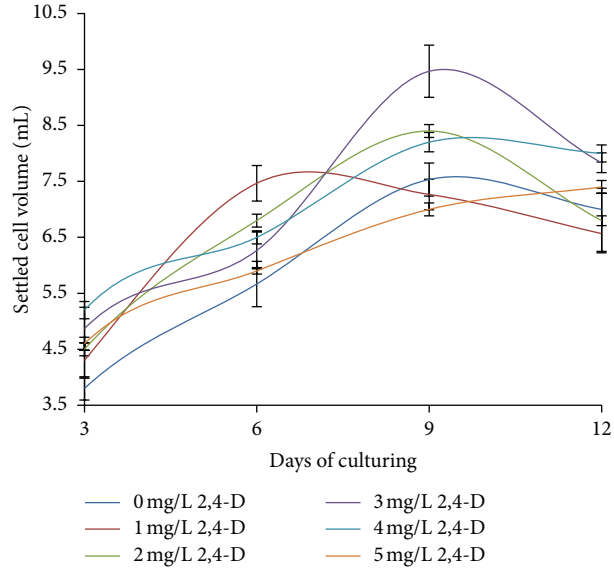

Figure 1: Effects of different concentrations of 2,4-D on the establishment of the *Mitragyna speciosa* cell suspension culture as measured by the settled cell volume (mL) in 25 mL of cell culture. The bars indicate the standard error.

and suspension culture of *Mitragyna speciosa*. After 12 days of observation, the 3% (w/v) sucrose treatment facilitated optimum growth as measured by SCV (9.33 ± 0.219 mL) (Figure 3). However, although the cultures that were treated with 3% and 4% (w/v) sucrose decreased after 9 days of culture, the 5% (w/v) sucrose treatment caused no sudden decline in the cell-growth pattern. Although the 3% sucrose treatment provided the highest SCV, the culture initially grew slowly when compared to the other treatments; however, its growth suddenly increased on day 9. To achieve optimum cell growth, different plants require different carbon source concentrations due to the plants' different enzymatic metabolism [27]. Therefore, the sugar availability should initiate different responses that affect plant metabolism, growth, and development.

3.3. Elicitation. To determine the effects of various concentrations of elicitors on mitragynine production, the cell suspension cultures were harvested six and twelve days after inoculation. A culture that lacked elicitors was used as the control. The mitragynine content was calculated using the calibration curve that was built with the Empower Software System (Figure 4). Different concentrations of yeast extract (100 mg L^{-1}, 250 mg L^{-1}, and 500 mg L^{-1}) were added to a 100 mL Erlenmeyer flask that contained 25 mL of WPM. Figure 5 shows the quantitative accumulation of mitragynine in response to different yeast extract concentrations at different harvesting times. The elicitation with yeast extract showed that the optimum mitragynine content was achieved with 250 mg L^{-1} yeast extract at six days of culture (9.275 ± 0.082 mg L^{-1}). This value is significantly different than that of the control. However, the elicitation with 500 mg L^{-1} yeast extract provided the lowest mitragynine content at six days of inoculation. This demonstrates that the "overloading" of

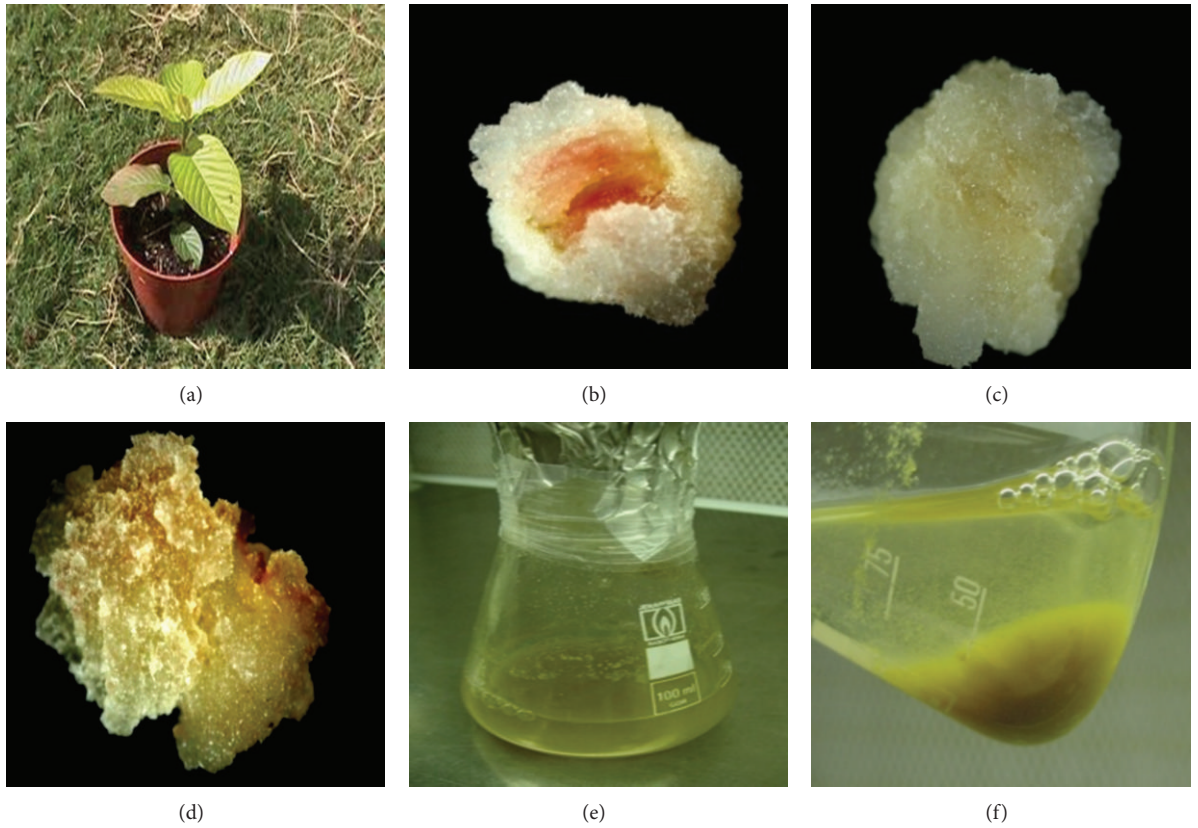

Figure 2: (a) *Mitragyna speciosa* tree, ((b)–(d)) callus of *Mitragyna speciosa*, and ((e)-(f)) *Mitragyna speciosa* suspension culture.

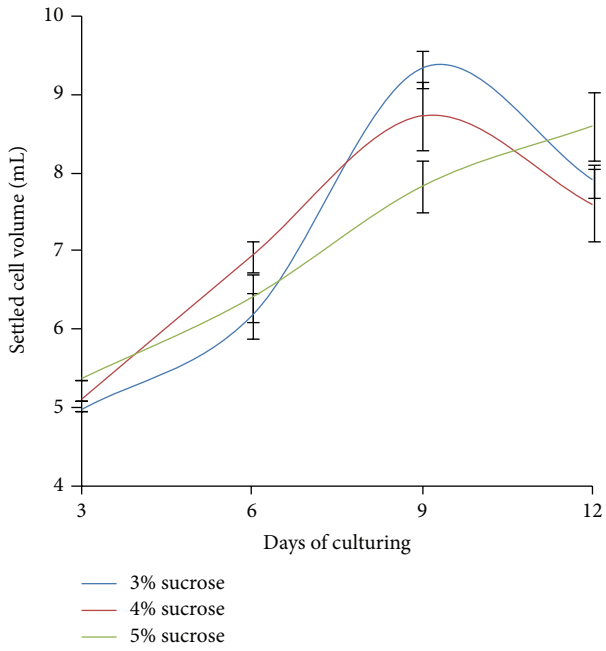

Figure 3: Effects of different sucrose concentrations of sucrose on the establishment of the *Mitragyna speciosa* cell suspension culture as measured by the settled cell volume (mL) in 25 mL of cell culture. The bars indicate the standard error.

the elicitor can have adverse effects. The phase at which the plant cell suspension was harvested also affected the product accumulation. The cultures that were harvested at twelve days of culture showed no significant differences in the mitragynine content among all of the treatments. The inoculation with the yeast extract for six days of culture more effectively induced the mitragynine content when compared to twelve days of culture (Figure 5). These differences demonstrate that the biochemical changes that are associated with the induction of secondary metabolism following the addition of elicitors are very complex; it is likely that different metabolic systems can be affected, and there is a large amount of biochemical alteration [28]. In other experiments, the elicitation with all of the salicylic acid treatments did not significantly affect mitragynine production with respect to the control (Figure 6). This result showed that salicylic acid cannot stimulate mitragynine production. Unsuitable elicitor concentrations may cause unsuccessful elicitation, which indicates that a successful elicitation is a challenging process that requires intense standardization [13]. In this study, salicylic acid was not found to be a suitable elicitor for the increased production of secondary metabolites, while it may be a suppressor that led to the low biomass production. The induction of secondary metabolites by an elicitor may cause an increased metabolite production and a decreased cell mass [29].

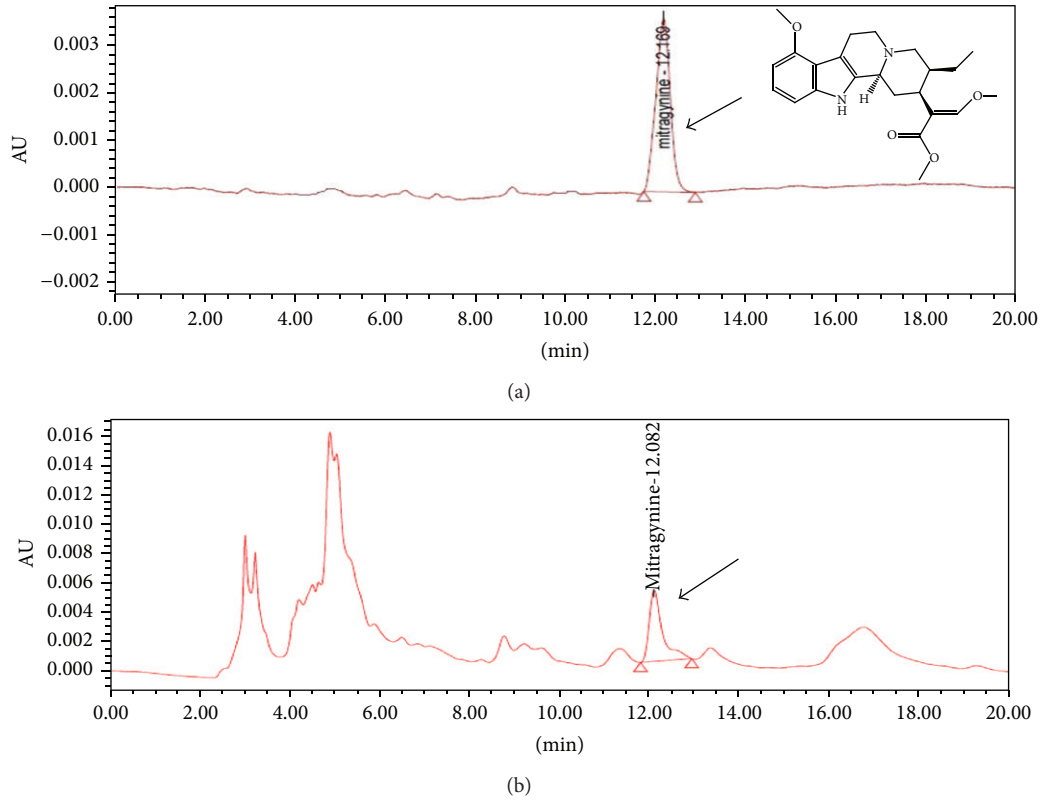

Figure 4: (a) HPLC chromatogram for the mitragynine standard and (b) chromatogram for the sample.

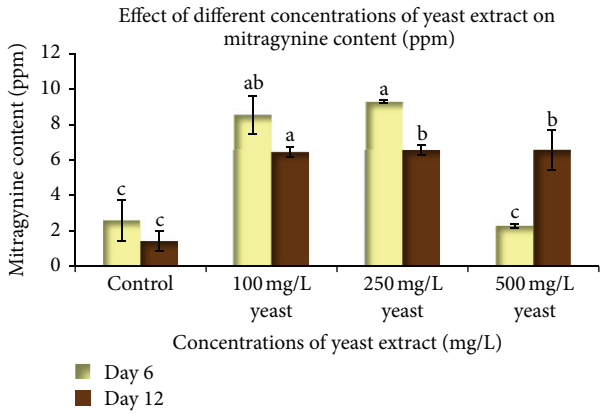

Figure 5: The effect of different yeast extract concentrations at different culture times on the mitragynine content in the *Mitragyna speciosa* suspension culture. The bars indicate standard error. *Values with the same letters are not significantly different according to Duncan's multiple range test.

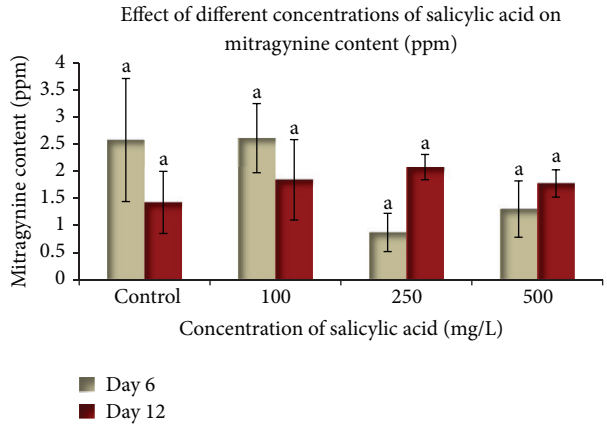

Figure 6: The effect of different salicylic acid concentrations at different culturing times on the mitragynine content in the *Mitragyna speciosa* suspension culture. The bars indicate standard error. *Values with the same letters are not significantly different according to Duncan's multiple range test.

3.4. Precursor Feeding. The optimization of culture conditions, such as medium composition and the addition of biosynthetic precursors, may enhance the production of secondary metabolites, which may ordinarily be restricted by the lack of precursors or enhancers [30]. Two types of precursors, tryptophan and loganin, were used in this experiment. Different concentrations were tested at different inoculation time periods to study their effects on alkaloid accumulation. These precursors were added to 25 mL of WPM that contained an *M. speciosa* suspension culture. The HPLC analysis showed that the optimum mitragynine content was achieved following a 3 μM tryptophan treatment at six days of culture (13.226 mg L^{-1}). This value was significant when compared to other treatments (Figure 7). The comparison

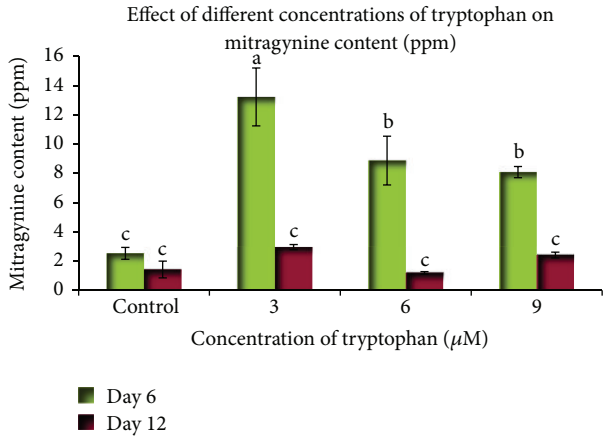

Figure 7: The effect of different tryptophan concentrations at different culture times on the mitragynine content in the suspension cultures of *Mitragyna speciosa*. The bars indicate standard error. *Values with the same letters are not significantly different according to Duncan's multiple range test.

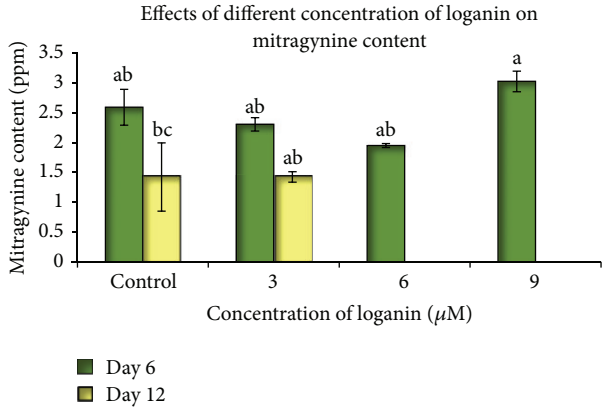

Figure 8: The effect of different loganin concentrations at different culture times on the mitragynine content in the suspension cultures of *Mitragyna speciosa*. The bars indicate standard error. *Values with the same letters are not significantly different according to Duncan's multiple range test.

between the treatment periods showed that the precursor inoculation for six days produced a better response when compared to twelve days of culture. In another experiment, the highest mitragynine content was obtained from the 9 μM loganin treatment that was harvested on day six of culture (3.028 mg L^{-1}), although this value was not significantly different among the treatments (Figure 8). In most of the treatments, the mitragynine content was lower than in the control. No mitragynine was detected following the treatment with either 6 μM or 9 μM loganin at twelve days of culture. This experiment showed that loganin is not a positive inducer of mitragynine content in *M. speciosa* suspension culture.

4. Discussion

The aims of this study were to develop a *M. speciosa* suspension culture system, to analyze the presence of mitragynine in the suspension cultures, and to use an elicitation and precursor feeding strategy to induce and increase mitragynine production in suspension culture. To obtain a good suspension culture, it is critical to initiate the suspension cultures from a friable callus source [31]. Callus is a dedifferentiated state of tissue that is achieved through the exogenous application of plant growth hormones *in vitro* [32]. Callus tissue is an essential material in plant cell culture systems. When a callus is introduced into a liquid medium and agitated, the cells disperse throughout the liquid to form a cell suspension culture. In theory, callus tissues are totipotent and have the potential to synthesize all of the compounds that are typically associated with an intact plant [33]. Therefore, an efficient procedure was developed for callus induction using different plant organs and different culture media that contained various hormone concentrations.

The results showed that leaf explants induced callus formation at a lower level than the petiole explants. The petiole explants showed a high efficiency of callus induction; this may have occurred because the tissues were more responsive to the supplied hormones. The *in vitro* proliferation of tissues depends on the application of phytohormones and on the ability of the tissues to respond to these hormonal changes during the culture [34]. The slow callus induction of the leaf explant culture may have been caused by an unsuitable nutrient supply that was not compatible with the ability of the tissues to respond. Indra [35] suggested that the genotype, the environment, and the developmental stage of explants greatly influence their growth and are critical for the development of *in vitro* culture. Because the petiole showed a highly responsive callus production, petiole explants were used to test the effects of different types and concentrations of hormones on callus induction.

The hormones that were used in this study were the combinations of 2,4-D and kinetin and BAP and NAA. The 2,4-D treatment alone without kinetin was found to have a higher organogenic potential and produced a loose friable, whitish-colored callus. The best results for callus proliferation and growth were achieved in a medium that contained 4.0 mg L^{-1} 2,4-D. As the friability of the cells increases, it is easier to achieve the full separation of the cells; this is ideal for the initiation of cell suspension cultures. Plant cell cultures are normally established and maintained on media that contain an auxin and a cytokinin. The removal of either hormone from the medium can result in the low induction of callus. This was observed in this study when WPM that lacked hormones (control) produced a low percentage of callus induction when compared to that with hormones. At the cellular level, auxins control basic processes, such as cell division and cell elongation. Because they can initiate cell division, they are involved in the formation of meristems that produce either unorganized tissue or defined organs. Cells that respond to auxin revert to a dedifferentiated state and begin to divide. To induce callus growth from the explants of dicotyledonous plants, a cytokinin is usually added to the

medium in addition to an auxin. A few studies have reported successful results from using kinetin in combination with 2,4-D to induce callus formation; for example, a combination of 0.5 mg L^{-1} 2,4-D and 0.05 mg L^{-1} kinetin was used to induce callus from *Arabidopsis thali*ana [36], 0.1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D induced callus in *Beta vulgaris* [37], and 5 mg/L 2,4-D and 5 mg/L kinetin induced callus in *Rudbeckia hirta* [38]. In the current study, kinetin, which is a cytokinin hormone, was used alone and in combination with auxin (2,4-D), but it did not stimulate a high percentage of callus induction when compared to 2,4-D alone (Table 2). This result indicates that kinetin cannot promote callus growth for *M. speciosa*. The application of 4 mg L^{-1} of the growth regulator 2,4-D is required to induce callus growth from petiole explants; however, kinetin did not support the role of 2,4-D to promote optimal callus growth. In another experiment, another combination of auxin and cytokinin (NAA combined with BAP) was tested on the promotion of callus proliferation. The result showed that 4 mg L^{-1} NAA that was combined with 0.5 mg L^{-1} BAP provided an optimum level of callus induction (50%) (Table 3). Using the Duncan multiple range test (DMRT), this result was significant with respect to the other treatments. The application of BAP or NAA alone did not promote callogenesis in the petiole segments in the *M. speciosa* cultures. The callus structure that was obtained from this experiment showed a globular shape with a white color, but the structure was hard. A hard and crusty callus structure is not suitable for the initiation of suspension cultures because it has a low dispersion and separation properties, and it is difficult to obtain single cells from a clump of cells. Therefore, the callus that was obtained from WPM that contained 4 mg L^{-1} 2,4-D was used to initiate *M. speciosa* suspension cultures. To prevent the callus cells from developing further, a cell suspension culture was established in which cell multiplication was the primary focus. The transfer of callus pieces into flasks that contain liquid media initiates the cell suspensions, in which the growth rates of the suspension-cultured cells in liquid media are generally higher than the growth rates on solid media. The liquid WPM that contained 3% sucrose and different concentrations of the plant growth regulator 2,4-D (1 to 5 mg L^{-1}) was tested to determine the best culture medium for establishing the suspension culture. The suspension was placed on a rotary shaker to provide aeration and to uniformly disperse the cells in the suspension. A culture that consisted of a high percentage of single cells and a high settled cell volume was considered to be a good suspension. After initiating the cell suspension with 2 g of initial inoculum, the cell volume slowly increased over the course of 3–6 days and eventually increased rapidly after 6 days of culture. After 9 days, the volume of cells gradually began to decrease. Immediately after they are cultured, cell division resumes after a lag phase, which leads to the exponential or logarithmic growth phase; during this phase, there is a biomass increase. Observations from the growth study showed that these cell suspensions can be maintained by performing sequential subcultures during the early stationary phase at a time when cell aggregation is maximal to avoid genetic variation in the suspension culture and to obtain a homogeneous population. Based on the cell behavior in the suspension culture that was grown in WPM containing 3 mg L^{-1} 2,4-D, the stage of subculturing was identified to be within 6–9 days; this was considered to be the critical subculturing stage to obtain the maximum production of secondary metabolites. Because all of the treatments showed a declining stage after 9 days of culture, the nutrients in the medium may have been exhausted and/or toxic metabolic byproducts may have been formed. Using liquid WPM that contained the best 2,4-D level and 3% sucrose, two additional percentages of sucrose (4 and 5%) were tested with regard to the cell viability percentage and settled cell volume as in the previous experiment. Sucrose is the primary energy source for *in vitro* plant tissue cultures because they have insufficient autotrophic abilities [39]. Sucrose (2–5%) is the most popular carbohydrate that is used in tissue culture. In general, most tissue culture studies are performed using sucrose as the sole carbon source due to its efficient uptake across the plasma membrane [40]. Sucrose acts as an external energy source and contributes to the osmotic potential of the medium, which facilitates the absorption of mineral nutrients that are present in the medium and are essential for cell growth; therefore, the optimal osmotic pressure is required for optimal proliferation [41].

A significant effect of the carbon source concentration on culture growth has been reported in many studies, such as those in rice [42], patchouli [40], *Gentiana kurroo* [43], olive [44], and *Melastoma malabathricum* [45]. In the current study, 3% sucrose was used as a control. The growth analysis status showed that the optimal sucrose level to obtain good suspension cultures is 30 gL^{-1} (3%). This result indicated that sucrose levels that were higher than 3% gave low efficiency of cell suspension volumes. The high sucrose content reduced the water content in the cultured cells, which may explain why the higher sucrose levels showed a slow cell growth trend in *M. speciosa*. This can be explained because the higher concentration of sucrose in the culture medium can reduce or slow the cell biomass due by increasing osmotic potential, which subsequently reduces nutrient uptake. The optimal liquid medium (3 mg L^{-1} 2,4-D and 3% sucrose) for the suspension cultures was then used as a basal medium in the elicitation and precursor feeding studies.

Two types of elicitors were used, yeast extract and salicylic acid, and two precursors were used, loganin and tryptophan. In this study, the optimum mitragynine concentration was achieved from the elicitation with 250 mg L^{-1} yeast extract in the cultures that were harvested at 6 days of culture (9.275 ± 0.082 mg L^{-1}). This concentration was 1.5-fold higher than in the cells that were harvested at 12 days of culture. However, the result was not significantly different when compared to the treatment with 100 mg L^{-1} yeast extract, which produced 8.523 mg L^{-1}. Both of the treatments were significantly different from the 500 mg L^{-1} yeast extract treatment. This result showed that at low concentrations of yeast extract higher concentrations of mitragynine were produced and at higher concentrations of yeast extract the mitragynine content was low. The type and concentration of the elicitor are critical to the elicitation process. A high elicitor dosage

may induce a hypersensitive that can lead to cell death, whereas an optimum level is required for induction [13]. This statement was supported by our results, in which 500 mg L^{-1} yeast extract produced a low mitragynine level. Reports have also described the use of different concentrations of yeast extract, such as the elicitation of sesquiterpenes in *Nicotiana tabacum* [46], the elicitation of sanguinarine in *Eschscholtzia californica* [47], the accumulation of alkaloids in *Eschscholtzia californica* suspension cultures [48], secondary metabolism in *Astragalus chrysochlorus* cell cultures [49], and the production of plumbagin in *Plumbago rosea* L. suspension cultures [50]. Furthermore, the elicitation of mitragynine using salicylic acid resulted in a low mitragynine concentration for all of the treatments, which was also generally lower than that in the control. Therefore, salicylic acid is not a good elicitor for elevating the production of mitragynine. Some reports have also shown unsuccessful results using salicylic acid as an elicitor, such as tropane alkaloid production in *Atropa belladonna* [51] and the production of taxol in suspension cultures of *Taxus chinensis* which was increased more significantly by methyl jasmonate than by salicylic acid [52].

Our precursor study showed that an optimum mitragynine level was achieved following a six-day 3 μM tryptophan treatment (13.226 mg L^{-1}); the level was 5-fold higher than the control. This result is significantly different among all of the treatments. Low mitragynine concentrations were observed in the cell suspension cultures that were harvested at 12 days of culture when compared to those that were harvested at 6 days. Higher tryptophan doses (6 and 9 μM) reduced the mitragynine concentration when compared to the 3 μM tryptophan dose. Although the specific process of mitragynine biosynthesis is still poorly understood, mitragynine is known to belong to the monoterpene indole alkaloid (MIA) group. Strictosidine is a common MIA that gives rise to more than 2,000 specific MIAs in different plant species [53]. Tryptophan is the starting material in the MIA biosynthetic pathway and is converted to tryptamine by the tryptophan decarboxylase enzyme. Tryptamine combined with secologanin by the presence of the strictosidine synthetase enzyme and yields strictosidine. Various enzymatic conversion reactions lead to the synthesis of various compounds from strictosidine [54]. To enhance the secondary metabolite production in cultured cells, it is possible to feed the precursor or one of the intermediates into the metabolic pathway so that it may be subsequently converted into a final product [55]. Therefore, the loganin precursor was also studied to determine its effect on mitragynine production. However, loganin did not positively affect mitragynine production (Figure 8), whereas the cell suspensions that were treated with 6 or 9 μM loganin that were harvested on day 12 did not produce any mitragynine. Therefore, instead of enhancing secondary metabolite production, loganin acted as an inhibitor. This may have resulted in low conversion of strictosidine from loganin and a secologanin.

Based on the limited knowledge of mitragynine biosynthesis, the trial and error method were used to improve the secondary metabolite content in a *Mitragyna speciosa* suspension culture. Nevertheless, these preliminary studies provided data regarding the construction of a tissue culture system, and the elicitation study in the *Mitragyna speciosa* suspension culture can be used as a guideline to provide a more appropriate protocol for further studies of this species.

Abbreviations

WPM: woody plant medium
2,4-D: 2,4-Dichlorophenoxy acetic acid
BAP: 6-Benzylaminopurine
NAA: 1-Naphthaleneacetic acid.

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Research Article

Annotation of Differentially Expressed Genes in the Somatic Embryogenesis of *Musa* and Their Location in the Banana Genome

Josefina Ines Maldonado-Borges, José Roberto Ku-Cauich, and Rosa Maria Escobedo-GraciaMedrano

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, A.C., C 43 No. 130, Col. Chuburná de Hidalgo, Mérida, Yucatán, C.P. 97200, Mexico

Correspondence should be addressed to Rosa Maria Escobedo-GraciaMedrano; escobedo@cicy.mx

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Analysis of cDNA-AFLP was used to study the genes expressed in zygotic and somatic embryogenesis of *Musa acuminata* Colla ssp. *malaccensis*, and a comparison was made between their differential transcribed fragments (TDFs) and the sequenced genome of the double haploid- (DH-) Pahang of the *malaccensis* subspecies that is available in the network. A total of 253 transcript-derived fragments (TDFs) were detected with apparent size of 100–4000 bp using 5 pairs of AFLP primers, of which 21 were differentially expressed during the different stages of banana embryogenesis; 15 of the sequences have matched DH-Pahang chromosomes, with 7 of them being homologous to gene sequences encoding either known or putative protein domains of higher plants. Four TDF sequences were located in all *Musa* chromosomes, while the rest were located in one or two chromosomes. Their putative individual function is briefly reviewed based on published information, and the potential roles of these genes in embryo development are discussed. Thus the availability of the genome of *Musa* and the information of TDFs sequences presented here opens new possibilities for an in-depth study of the molecular and biochemical research of zygotic and somatic embryogenesis of *Musa*.

1. Introduction

Somatic embryogenesis is a powerful tool for the massive production of elite plant materials, as well as for molecular agricultural breeding through the use of biotechnological strategies. Although this technology can be applied to any plant species, it is particularly valuable for the asexually propagated ones, such as bananas (*Musa* spp.). Bananas are one of the most consumed fruits worldwide and represent an important source of revenue for tropical countries where they also account for one of the main staple foods. In spite of their nutritional and socioeconomic importance, molecular tools for genetic improvement of bananas are limited in comparison to other plant species. In addition, the molecular bases of zygotic and somatic embryogenesis in *Musa* are not fully understood.

Recently, the genomic sequence of the double haploid banana-Pahang CIRAD 930 ITC 1511 (hereafter ITC 1511) was recently released [1]. ITC 1511 is derived from the Pahang wild diploid ($2n = 22$) *Musa acuminata* Colla. ssp. *malaccensis* accession which shares its genetic lineage with dessert and cooking bananas. The DH-Pahang genome size is 523 Mb (1C estimated through flow cytometry), and in a 91% assemblage it revealed 36,542 protein-coding genes anchored to the 11 *Musa* chromosomes. This provides a unique platform for genetic improvement of this underresearched vital crop. Besides the protein-coding genes, those for 235 microRNAs (*MIR*), corresponding to 37 different families, were found, including the eight families typical of Poaceae [1].

Using *M. acuminata* ssp. *malaccensis* immature zygotic embryos (IZE) we have developed an efficient somatic embryogenesis protocol, based on modifications to the one previously reported [2]. Moreover, we have observed changes in gene expression patterns during this process. Here, taking advantage of the information made available

regarding the *Musa* genome, we were able to assign putative functions to some of these genes as well as to localize their position into the chromosomes of the *Musa* genome available. Our finding suggest that the cDNA-AFLP procedure was useful for identifying expressed genes during early and late zygotic and somatic embryogenesis in *M. acuminata* ssp. *malaccensis* and helping annotate them in the *Musa* genome, such as cytidine triphosphate synthase 2 (CTP synthase 2), serine/threonine protein kinase, starch branching enzyme (SBE1), early responsive to dehydration (ERD) and indole-3-acetic-acid-amido synthetase (GH3.1); additional work in this line is underway.

2. Material and Methods

2.1. Plant Material and Establishment of Somatic Embryogenesis. In our study immature and mature zygotic embryos of *Musa acuminata* ssp. *malaccensis* (ITC 250) were obtained from fruits harvested from CICY's collection, located at the Experimental Station of the Instituto Nacional de Investigaciones Forestales (INIFAP) in Uxmal (Yucatán, Mexico; 20°21′34″N 89°46′17″W).

Embryogenic callus cultures were initiated from immature zygotic embryos (IZE) from fruits collected 60 to 65 days after-anthesis (DPA); embryos were extracted aseptically and cultured as described by Navarro et al. [2], except that for induction medium (MI) auxin was reduced to 4.5 mM of 2,4-dichlorophenoxyacetic acid (2,4-D). After three months in culture, cell suspensions were initiated from embryogenic calli in Cote liquid media M2 [3] and equal concentration of 2,4-D. Cultures were kept in the dark, in a shaker at 90 rpm, with media refreshed every two weeks. For embryo development, five-day-old cell suspensions were sieved through 60-mesh screen (230 μm), and 250 μL of the homogeneous embryogenic cell suspension (ECS), representing a 3% packed cell volume (PCV), was overlaid onto maturation media (MM) lacking plant growth regulator, either in agar or a disc of filter paper (Whatman no. 1, 9.0 cm in diameter) [2] in Petri dishes (100 × 15 mm) for a period of 45 to 60 days. Embryos were classified according to development as immature (globular in shape of whitish translucent appearance) and mature (torpedo-like, white opaque with a cotydelonary slit). Somatic mature embryos were germinated on Murashige and Skoog [4] media (MG) using 2.0 μM 6-BA and 2.85 μM IAA [2].

2.2. Collection of Zygotic and Somatic Embryogenesis Stages for RNA Extraction. For this study immature (between 60 and 65 dpa) and mature (between 90 and 100 dpa) zygotic embryos were collected along with immature and mature somatic embryos. The different developmental stages were defined by histological [2] and scanning electron microscopy (SEM) assessment [5] (see Figure 2). Tissues at different developmental stages (immature zygotic (IZE), mature zygotic (MZE) embryos, embryogenic callus (EC), immature somatic (ISE), and mature somatic (MSE) embryos) were collected and rapidly freezed in liquid nitrogen, and samples were kept at −80°C until used for RNA extraction.

2.3. Differential Gene Expression. Gene expression at the different developmental stages of zygotic and somatic embryos (Figure 2) was analyzed by cDNA-AFLP. Total RNA from the different embryogenic phases was prepared using trizol reagent (Invitrogen, USA) and after some adjustments followed the protocol of Chomczynski and Saccchi (1987). RNA extracts were treated with RNase-free DNase I (Invitrogen), and first-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using random primers. cDNA-AFLP was performed according to Vos et al. [6] and Bachem et al. [7], with modifications and using AFLP primers set (Table 1). The selective amplification primers were selected based on the high polymorphism previously shown for different banana species, including *M. acuminata* ssp. *malaccensis* [8]. cDNA was first digested with Mse I and then with Eco RI. Adaptors for both enzymes were then ligated to the extremes of the restriction fragments, in order to generate the substrates for amplification. Twenty rounds of preamplification were performed using AFLP primers with selective nucleotides (**C** for MseI and **A** for EcoRI, resp.). Reaction mixture (25 μL) was prepared with 2.5 μL of 10x PCR buffer, 0.75 μL of 50 mM $MgCl_2$, 0.75 μL of each 30 μM primer solution, 2.5 μL of a 2 mM dNTPs, 5 μL of the cDNA ligated and 1 : 10 diluted, and 0.125 μL (5U) of Taq-DNA pol (Invitrogen). PCR cycles were at 92° (1 min), 56° (30 seg), and 72°C (1 min) for denaturalization, primer alignment, and amplification, respectively. Products were diluted (1 : 10), and 5 mL was amplified using five different primer combinations (Figure 2). These combinations already detected a high degree of polymorphism in *M. acuminata* ssp. *malaccensis* [8]. Reaction mixtures were prepared as described previously, but 0.38 μL of 20 μM primer solutions was added. PCR cycles (35) were at 94° (30 seg), 65° (30 seg), and 72°C (1 min) for denaturalization, primer alignment, and amplification, respectively. PCR products were mixed with an equal volume of loading buffer (0.01% bromophenol blue, 0.01% xylene cyanol, and 10 mM EDTA in 98% formaldehyde, pH 8.0), denatured at 95°C, and then kept in ice. Samples were electrophoresed in 6% polyacrylamide denaturing gels with TBE 1.0x (89 mM Tris pH 7.6, 89 mM boric acid, and 2 mM EDTA), at 55 W, 2000 V, 50 mA. Gels were stained with silver nitrate [9], dried, and digitalized for band analysis. The presence or absence of differential bands was registered for the different developmental stages and primer combinations (Figure 2).

2.4. Transcript-Derived Fragment (TDF) Isolation and Reamplification. The differentially expressed TDFs were assigned based on presence, absence, or differences of intensity and were cut with a sharp blade from the gel with care to avoid contaminations prior elution in 25 μL of PCR buffer 2x. Aliquots of 2 μL were reamplified as described previously using the same set of primers and PCR conditions as used for preamplification. Amplicons were resolved in 1.2% agarose gels; each single band was isolated and eluted using the QIAEX II Gel Extraction package (QUIAGEN).

2.5. Cloning and Sequencing of TDFs. Eluted TDFs were cloned into the plasmid pGEM-T Easy vector (Promega,

Table 1: Set of AFLP primer and adaptor pairs used for the ligation: preselective and selective amplification of cDNA (in bold: selective nucleotides, PA: preselective primers for amplification).

Primer pair	Adapter/primer	Sequence 5′-3′	
	Adp.1 eEcoR1	CTCGTAGACTGCGTACC	
	Adp.2 eEcoR1	AATTGGTACGCAGTCT	
	Adp.1 eMse I	GACGATGAGTCCTGAG	
	Adp.2 eMse I	TACTCAGGACTCAT	
	PA-eEcoRI-01	GACTGCGTACCAATTCC	
	PA-eMse I-02	GATGAGTCCTGAGTAAC	
		EcoR1, 3+	**Mse I, 3+**
(1) E4-M1		GACTGCGTACCAATTC**ACG**	GATGAGTCCTGATAA**CAA**
(2) E1-M3		GACTGCGTACCAATTC**AAC**	GATGAGTCCTGATAA**CAG**
(3) E4C-M10		GACTGCGTACCAATTC**CAC**	GATGAGTCCTGATAA**CCG**
(4) E7C-M10		GACTGCGTACCAATTC**CGC**	GATGAGTCCTGATAA**CCG**
(5) E15C-M10		GACTGCGTACCAATTC**CTC**	GATGAGTCCTGATAA**CCG**

Madison, WI, USA) and used to transform *E. coli* DHα5 cells. The cloned cDNA fragments were sequenced using a commercial service (Macrogen Inc., Seol, Republic of Korea). Sequences of TDF were cleaned by trimming off the plasmid sequences and then analyzed for homology against the NCBI database. TDFs were also compared to the *Musa* genome database (http://banana-genome.cirad.fr; [1]) to assign putative identities and function.

3. Results

cDNA-AFLP analysis of the RNA samples from immature and mature zygotic and somatic embryos stages as well as embryogenic cell suspension culture materials of *M. acuminata* ssp. *malaccensis* (Figure 2) with five pairs of primers (Table 1) resulted in the identification of a total of 253 TDFs with a range in size from 100 to 4000 bp. Of these, 21 TDFs were clear and unambiguously differentially expressed through the process of banana embryogenesis (see Figures 1(a)–1(f), and Figure 2). These 21 TDFs differentially expressed ranged from 76 to 299 bp. Comparisons of the *Musa* DH-Pahang genome allowed to assign location of 15 of them on the *Musa* chromosomes, as well as possible identity (Table 2). Seven of these sequences corresponded to typical protein domains of higher plants and *Musa*. Interestingly, four sequences were located in all the *Musa* chromosomes, and two of them, namely, 17-1-5 and 47-1-4, were found only in mature embryos. The former of them occurred both in MSE (Figure 1(f), line 5) and MZE (Figure 1(b), lines 4 and 5), whereas the latter was only detected in MSE. The last two TDFs (24-2-1 and 46-5-4) occurred at all stages of the embryogenesis process, regardless of their origin, zygotic or somatic (Table 2). The remaining eleven sequences were located in either one or two chromosomes; six of these sequences were only found in ECS.

4. Discussion

The understanding of the type and number of genes differentially expressed during embryogenesis in *Musa* would help discerning the molecular mechanisms involved during the passage through the different stages involved in the processes, both of zygotic and somatic origins. It also opens the path towards biotechnological fundamental studies. To our knowledge this work represents one of the first steps in that direction. The results shown here contribute to the allocation of the putative function and participation of genes identified during the sequencing of the DH-Pahang *Musa* genome [1].

In this study, somatic embryogenesis induced *in vitro* and zygotic embryogenesis from collected stages of fruits with seeds of *M. acuminata* ssp. *malaccensis* plants allowed the identification of genes expressed during both important processes. Important differences in TDFs were observed among stages of zygotic and somatic embryogenesis of *M. acuminata* ssp. *malaccensis*. TDFs differential patterns corresponded to genes involved both in primary and secondary metabolisms, signal transduction, gene regulation, energy metabolism, and defense and cellular processes. Out of the 253 differential TDFs, only 15 could be located on the chromosomes and showed between 88 and 100% identity to available *Musa* genome sequences, thereby suggesting that most of the TDFs in the current study represent genes of banana related to embryogenesis.

Interestingly, TDFs of cytidine triphosphate synthase 2 (CTPS-2) were found in mature zygotic embryo as well as in proembryo and embryogenic cells suspensions. This protein is involved in the metabolism of pyrimidine and it has not been studied in depth within plants, although five gene copies encoding this protein have been identified in *Arabidopsis* [10]; two cDNAs were found to be upregulated during the ripening of apples [11]. Pyrimidine, like purine nucleotides, represents fundamental compounds, central to both primary and secondary plant metabolisms. Since it is involved in different cellular processes, pyrimidine is considered of vital importance for plant growth, development, and reproduction, during germination, pollen tube growth, flowering, and seed formation. Moreover, carbohydrate metabolism is closely linked to pyrimidine nucleotides since many enzymes involved in carbohydrate interconvention require

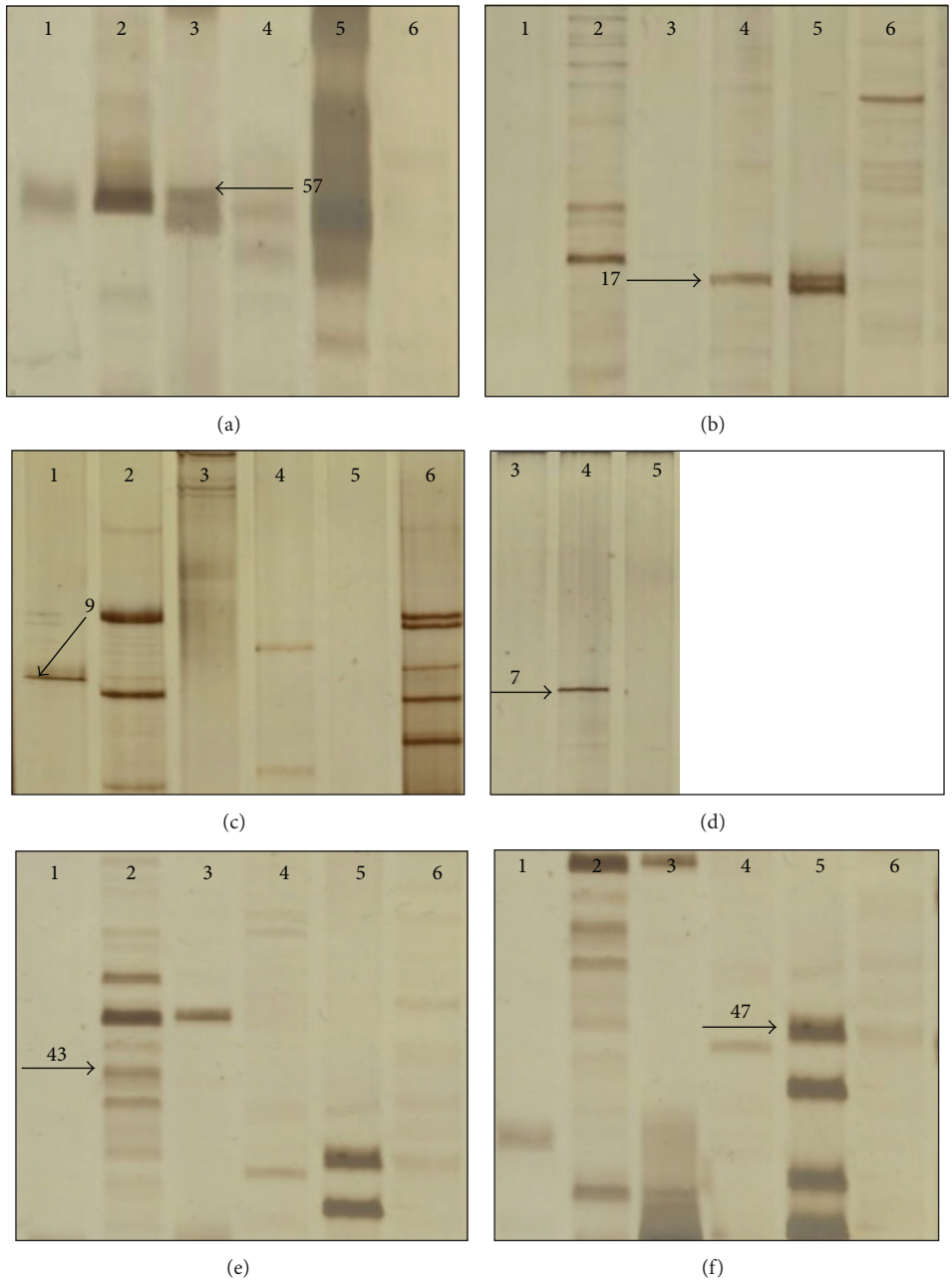

Figure 1: cDNA-AFLP patterns: zygotic versus somatic embryogenesis stages of *M. acuminata* ssp. *malaccensis*. Arrows with numbers showed TDF differentially expressed. (a) Early embryogenesis, (b) late embryogenesis, (c) immature zygotic embryo, (IZE), (d) mature zygotic embryo (MZE), (e) immature somatic embryo (ISE), and (f) matured somatic embryo (MSE). The numbers are 1: IZE, 2: ISE, 3: ECS, 4: MZE, 5: MSE, and 6: germinated somatic embryo (GSE).

this nucleotide as a substrate. Besides the synthesis of important cellular metabolites such as cell wall polysaccharides and glycoproteins, glycolipids and sulfolipids require pyrimidine nucleotides for their production; therefore a regulatory link between the levels of pyrimidine nucleotides and a large number of cellular biochemical processes require to be further explored [10].

TDFs representing genes for protein serine/threonine kinase involved in signal transduction were found in proembryos and embryogenic cells from suspension cultures. Protein phosphorylation, catalyzed by protein kinases, is one of the most fundamental regulatory mechanisms known to control protein activity and cellular signaling [12]. The network of these proteins in the plant cell appears to act as a "central processing unit" which accepts the receptors' information, recognizes changes in environmental conditions, that is, plant growth regulators, and other external factors, and converts the information into a suitable signal such as changes in metabolism, gene expression, and cell growth and division [13].

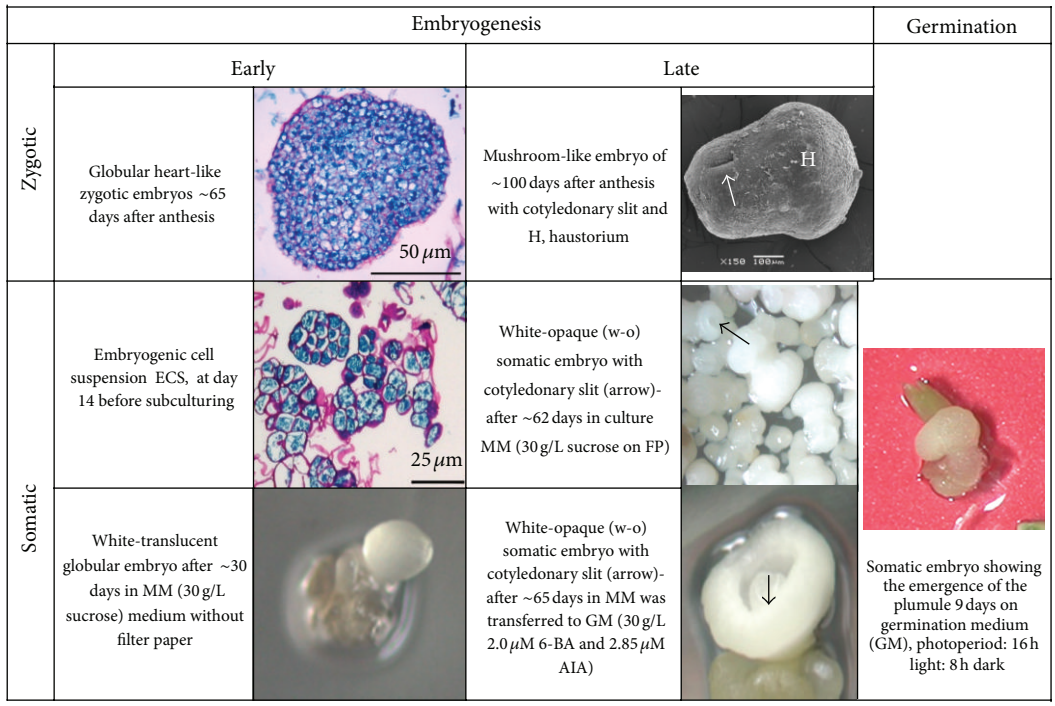

Figure 2: Different stages of zygotic and somatic embryogenesis of *M. acuminata* ssp. *malaccensis* plant material used for cDNA-AFLP analysis.

Table 2: Annotation of differentially expressed genes during different stages of somatic versus zygotic embryogenesis in *Musa acuminata* ssp. *malaccensis* and their location in the chromosomes of banana genome.

TDF number (base pairs)	Hit to the *Musa* chromosome no.	*E* value	% identity	Protein	Embryogenesis stage
7-1-2 (88)	6	9*E* − 21	95.24	CTP synthase 2	MZE
7-1-4 (71)	5	4*E* − 13	97.62	Serine/threonine-protein kinase	ECS
17-1-5 (299)	All	2*E* − 55	99.12	—	MSE, MZE
24-2-1 (286)	6	5*E* − 56	99.0	1,4-α-glucan-branching enzyme 2 chloroplastic/amyloplastic ~SBE1	All stages of somatic embryogenesis
35-2-3 (225)	1	1*E* − 107	99.5	—	IZE
43-1-1 (163)	10	8*E* − 66	99.24	—	IZE
46-5-4 (119)	All	2*E* − 22	89.36	Putative signal peptide peptidase-like 3	All stages of somatic embryogenesis
47-1-2 (148)	7	3*E* − 59	100	—	MSE
47-1-4 (149)	All (6)	3*E* − 25	87.83	—	MSE
47-1-5 (192)	4	8*E* − 11	92.16	Early-responsive to dehydration protein-related	MSE
58-1-2 (94)	4	6*E* − 16	96.08	Early-responsive to dehydration protein-related,	ECS
58-1-3 (88)	6	9*E* − 21	95.24	CTP synthase 2	ECS
60-1-3 (71)	5	4*E* − 13	97.62	Serine/threonine-protein kinase	ECS
60-5-4 (120)	6	3*E* − 18	90.67	Putative BSD domain containing protein	ECS
60-5-7 (116)	7	2*E* − 44	100	Probably the indole-3-acetic-acid-amido synthetase GH3.1	ECS

MZE and MSE: matured zygotic and somatic embryo, IZE: immature zygotic embryo; ECS: embryogenic cell suspension.

In addition, TDFs with differential expression for starch branching enzyme (1,4-α-glucan-branching enzyme 2, chloroplastic/amyloplastic ~(SBE1)), an enzyme that participates in starch metabolism, indicate its involvement in all stages of somatic embryogenesis in *Musa*. Starch is the major carbohydrate reserve of plant cells. The synthesis of amylopectin, one of the two major components of starch, is controlled by the activity of enzymes of three components: the starch synthase, starch branching, and starch disbranching enzymes [14]. Starch branching enzyme (SBE) plays an important role in starch biosynthesis by introducing branch points, the α-1,6 linkages in starch. Studies in maize indicate that different isoforms of *Sbe* are independently controlled; that is, *Sbe2b* appears to be endosperm specific, whereas *Sbe2a* form is at high levels in embryo than endosperm, and the absence of *(SBE1)* is associated with altered physiological function of starch [15]. In our case, we did not detect TDFs for starch branching enzyme during stages of zygotic embryogenesis, though it is possible as in maize that similar events could be related to the different zygotic embryo stages, a fact that merits further study, and the role that this enzyme(s) is taking during somatic embryo development. Also during somatic embryogenesis TDFs for the signal peptide peptidase-3 were detected; the signal peptide peptidases (SPP) are members of a family of proteases that are responsible for intramembrane processing of other proteins during the intracellular signaling events. In *Arabidopsis thaliana* six genes encoding these proteins were found; their physiological functions are not fully known [16], but it seems to require for male gametophyte development maturation of the pollen and its germination. In *Musa*, the physiological role of the signal peptide peptidase and their substrates continues to be unknown.

TDFs related to early-responsive to dehydration (ERD) proteins were found in mature somatic embryos and embryogenic cell suspension (MZE and ECS) cultures of *M. acuminata* ssp. *malaccensis*. The *ERD* genes are defined as genes rapidly activated during drought stress. The encoded proteins show great structural and functional diversity and are the first line of defense against drought stress in plants [17]. To date, a total of 16 complementary DNA (cDNA) for *ERD* genes have been isolated from *Arabidopsis thaliana,* and only half of these have been characterized in soybean. Such genes encode proteins that include ClpA/B ATP-dependent protease, heat shock proteins HSP-70-1, methionine-dependent methyltransferases, membrane proteins, proline dehydrogenase, carbohydrate transporters, senescence-related genes, glutathione-S transferase type LEA proteins (Late Embryogenesis Abundant), jasmonic acid biosynthesis proteins, chloroplast proteins, and hydrophilic and extension ubiquitin proteins. Regarding the expression controlled by phytohormones ERD genes have several functions in response to ABA signaling during germination and development and/or are involved in stress tolerance. Some genes may be induced in response to more than one phytohormone. The common characteristic of these genes is that their expression is increased rapidly in response to environmental stress; it is also suggested that such genes may function to regulate the expression of effectors' proteins and signaling pathways in response to stress [18]. In our case the presence of TDFs related to ERD was consistent with MSE; during SE maturation there was a restriction of water availability owing to the use of a filter paper between the developing embryos and the culture medium, perhaps with an increase in the hormone ABA due to drought stress imposed prior to germination, while in embryogenic cell suspension cultures probably the osmotic pressure by sucrose in the medium and/or the concentration of the added exogenous auxin act as abiotic stress that result in the expression of the TDFs related to ERDs.

In ECS of *M. acuminata* ssp. *malaccensis* we found TDFs recognized as BSD-domain containing proteins, which belong to a family of transcription factors, TFs. The BSD domain is characterized by three α helices, probably involved in DNA binding, and by conserved tryptophan and phenylalanine residues located at the C-terminus of the domain [19]. The BSD domain is associated with basal transcription factors, proteins linked with synapses, and different hypothetical proteins present in a variety of species ranging from protozoans to humans [20]. Thus it is likely that the BSD-domain containing protein found here represents basal TFs associated with cell proliferation during somatic embryogenesis *Musa* as well as other plant species.

In this study TDFs differentially expressed in ECS of *Musa* were found to be related to the indole-3-acetic acid-amido synthetase (GH3.1) (Aux/IAA, amido synthetase, GH3) gene, such protein is also called auxin-responsive GH3-like protein, it is involved in the catalysis of the synthesis of IAA-amino acid conjugates, providing a mechanism for the plant to cope with the presence of excess auxin. Maintaining homeostasis through converting free IAA to IAA conjugated with carbohydrates, amino and methyl groups forms are a conserved mechanism in monocots and dicots. The GH3 family proteins are responsible for converting the active IAA to its inactive form by conjugating the amino acid free IAA. The members of this gene family in *Arabidopsis* are regulated by hormones and environmental factors, including salicylic acid, abscisic acid, and light pathogen infection [21]. Auxin regulates the growth and development of plants by altering the expression of various genes, including genes such as GH3 widely studied in dicots, but little information is available in monocots. In rice 12 members of GH3 family genes have been identified; transcripts abundance increased by auxin treatments, sustaining a role in signal transduction pathway [22]. In cell suspension cultures where we assume that this enzyme was produced by the presence of auxin during culture and has been associated with this signaling pathway, further work will elucidate their role in banana embryogenesis.

In conclusion, the procedure of cDNA-AFLP was useful for identifying genes expressed during early and late zygotic and somatic embryogenesis in *M. acuminata* ssp. *malaccensis*, compared the events, and annotated their location in the available DH-Pahang sequence genome. This represents a contribution to the known genetic changes that lie behind this process in monocotyledons since the allocation of genes not currently recognized as involved in such biological processes or metabolic pathways is suggested. Additionally, we have identified a number of TDFs with significantly lower

expression levels in ESC and IZE; these could encode an interesting candidate proteins involved in embryogenesis.

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Research Article

Genetic Diversity Analysis of Sugarcane Parents in Chinese Breeding Programmes Using gSSR Markers

Qian You, Liping Xu, Yifeng Zheng, and Youxiong Que

Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, China

Correspondence should be addressed to Liping Xu; xlpmail@126.com and Youxiong Que; queyouxiong@hotmail.com

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Sugarcane is the most important sugar and bioenergy crop in the world. The selection and combination of parents for crossing rely on an understanding of their genetic structures and molecular diversity. In the present study, 115 sugarcane genotypes used for parental crossing were genotyped based on five genomic simple sequence repeat marker (gSSR) loci and 88 polymorphic alleles of loci (100%) as detected by capillary electrophoresis. The values of genetic diversity parameters across the populations indicate that the genetic variation intrapopulation (90.5%) was much larger than that of interpopulation (9.5%). Cluster analysis revealed that there were three groups termed as groups I, II, and III within the 115 genotypes. The genotypes released by each breeding programme showed closer genetic relationships, except the YC series released by Hainan sugarcane breeding station. Using principle component analysis (PCA), the first and second principal components accounted for a cumulative 76% of the total variances, in which 43% were for common parents and 33% were for new parents, respectively. The knowledge obtained in this study should be useful to future breeding programs for increasing genetic diversity of sugarcane varieties and cultivars to meet the demand of sugarcane cultivation for sugar and bioenergy use.

1. Introduction

Sugarcane (*Saccharum* spp.) is the main sugar and bioenergy crop in the world. In comparison to other countries, Chinese sugar consumption is much lower and has only about 1/3 average of the world due to the different diet. However, the total sugar consumption, production, and import are in the second, third, and first positions in the world in recent years [1]. In addition, sugar from sugarcane occupies about 90%–92% of the total sugar output in China [2]. With an increasing demand for sugar, sugarcane shows more potential in China, leading to over one million sugarcane seedlings cultivated, which are produced from a total of 600–700 cross combinations every year in China [1]. The security of sugarcane cultivation is under threat from a number of diseases, especially smut disease caused by *Sporisorium scitamineum* and mosaic disease caused by *sugarcane mosaic virus* or *sorghum mosaic virus*. This leads to a demand for heterogeneity of cultivars. However, the heterogeneity of cultivars remains low, since the three "ROC" serial varieties account for about 85% of the total sugarcane cultivated area in China, with one (ROC22) responsible for about 50%–60% of the cultivated area in the last ten years [1]. Cross breeding is the most important way for breeding new sugarcane varieties and variety improvement, and it has played a significant role in the development of sugar industries in almost all the sugarcane-producing countries [3]. In addition, parental crosses of sugarcane always improve significantly the cane stalk yield and sugar content; thus, it is important to get the understanding of the genetic diversity of parents for crosses in breeding programs in China.

Traditional ways for sugarcane breeders to identify the relationships among varieties rely on anatomical and morphological characters [4]. In recent years, genetic diversity has been investigated for sugarcane cultivars or ancestral species by using several molecular methods, such as restriction fragment length polymorphism (RFLP) [5, 6], random amplified polymorphic DNA (RAPD) [7, 8], amplified fragment length polymorphism (AFLP) [9], intersimple sequence repeats (ISSR) [10, 11], sequence-related amplified polymorphism (SRAP) [12, 13], target region amplification polymorphism (TRAP) [14, 15], genomic in situ hybridization

(GISH) [16, 17], fluorescence in situ hybridization (FISH) [17–19], genomic simple sequence repeats (gSSR, hereinafter referred to as SSR) [9], and expressed sequence tag-SSR (EST-SSR) markers [20]. Among all the above molecular techniques, SSR markers are widely used in the genetic diversity analysis of sugarcane because they are codominantly inherited, abundant, and highly reproducible [20–22]. Cordeiro et al. (2003) used six gSSR markers to assess the genetic diversity level between the 66 accessions which included the genera Saccharum (*S. officinarum*, *S. spontaneum*, and *S. sinense*), Old World *Erianthus* Michx. sect. *Ripidium*, North American *E. giganteus* (*S. giganteum*), *Sorghum,* and *Miscanthus* [23]. Liu et al. (2011) and Pan (2010) used polymorphic SSR DNA markers to genotype sugarcane clones with a fluorescence electrophoresis (CE)-based genotyping system [24, 25]. A few studies have also been reported on the genetic diversity of sugarcane parental accessions by SSR markers [26, 27].

Some accessions have played a particular key role in the development of commercial sugarcane varieties and thus have been designed as common breeding parents [28, 29]. In addition, new parental materials are more important for broadening genetic basis in the development of modern varieties used for cultivation and breeding [30, 31]. Therefore, investigation of the genetic relationships among common and new parental accessions is necessary for future sugarcane improvement and breeding in China.

In sugarcane breeding programmes, the choice of parents for crossing largely depends on the aims and objectives of the breeder. In the past, this was generally based on phenotypic and genotypic expression of the characters they display and especially on the superior progeny, that is, the potential ability of cane sugar yield of varieties derived from the cross combinations, which is also influenced by the environment and a series of uncontrolled factors. The objective of the present study is to evaluate the genetic diversity of 115 sugarcane cross parents, termed as common or new parents, using SSR markers. For the molecular analysis, two levels of analysis were investigated. Firstly, the within and between population diversity was evaluated on 64 common parents and 51 new parents, each represented by different groups, and the genetic parameters between the two groups of accessions were analyzed, respectively. Secondly, cluster analysis by unweighted pair group method with arithmetic mean (UPGMA) and principle component analysis (PCA) of 115 parents was performed. The information obtained in this study will be valuable for choice of parents and cross prediction and especially for the development of cultivar improvement programs in modern sugarcane breeding.

2. Materials and Methods

2.1. Plant Materials. The background of the sugarcane parents used in this study was given in Table 1. Leaf samples of a total of 115 sugarcane accessions, including 64 common parents and 51 new parents, were collected. They were cultivated in Sugarcane Resources Nursery of FAFU (Fujian Agriculture and Forestry University, Fuzhou, China) and Ruili Breeding Station in Yunnan Academy of Agriculture Science (Ruili, Yunnan, China).

2.2. DNA Extraction. DNA extractions from the leaf tissues were conducted according to biospin plant genomic DNA extraction kit specification (Bioflux, Japan). Each leaf sample was collected from three independent sugarcane plants and only +1 leaf from each plant. After detection of the quality and concentration, this batch of genomic DNA was diluted to a suitable concentration and stored at −20°C.

2.3. SSR Analysis. A total of five highly polymorphic SSR DNA markers (SMC334BS, SMC336BS, SMC36BUQ, SMC286CS, and SMC569CS) were selected from 221 ICSB sugarcane SSR markers [24, 32]. Forward primers of all these SSR primers were labeled with FAM, the fluorescence dye. PCR amplification was performed in a 25 μL reaction containing 50 ng of genomic DNA, 2.5 μL 10 × PCR buffer, 0.2 μM of each primer, 200 μM dNTP mixtures, and 1.0 U of *rTaq* polymerase. PCR comprised the following steps: the first cycle was preceded by a 3 min denaturation at 94°C, then thirty-one PCR cycles were performed in a PCR amplifier (Eppendorf 5333), with each cycle consisting of denaturation at 94°C for 30 s, annealing at either 58°C, 60°C, 62°C, or 64°C for 30 s (SMC286CS, SMC334BS, SMC569CS, and SMC36BUQ) and 62°C for 35 s (SMC336BS), and extension at 72°C for 30 or 35 s, and the last cycle was followed by a 2 min final extension at 72°C. Fragment analyses of amplified PCR products were conducted by capillary electrophoresis (CE) on ABI PRISM 377-96 DNA sequencer (Applied Biosystems) according to the manufacturer's instructions. Each CE sample included 1.0 μL post-PCR reaction mixture, 0.5 μL of ROX-360 size standards, and 8.5 μL loading buffer of which the major ingredient contained polyacrylamide and dextran-blue. Then, PCR-amplified SSR DNA fragments were separated, and both the size standard and PCR amplified fragments were recorded automatically into individual GeneScan files.

2.4. Data Analyses. The data obtained from GeneScan files were analyzed with GeneMapper software (Applied Biosystems) to produce capillary electropherograms of amplified DNA fragments. GeneMapper parameters were set as follows: plate check module: Plate Check A; prerun module: GS PR36A-2400; run module: GS run 36A-2400; collect time: 2.5 h; and lanes: 64. An SSR allele or peak was scored either as present (1) or absent (0), except for "stutters," "pull-ups," "dinosaur tails," or "minus adenine" [24, 32]. The polymorphic information content (PIC) was calculated by the formula $\text{PIC} = 1 - \sum P_i^2$, where P_i is the frequency of the population carrying the ith allele, counted for each SSR locus [21]. Then, the binary data matrices were used for genetic diversity parameter analysis. POPGENE 1.31 [33] was used to determine number of polymorphic bands (NPB); percentage of polymorphic bands (PPB); observed number of alleles (Na); and effective number of alleles (Ne). Nei's genetic diversity (h), mean values of total gene diversity (Ht), and Shannon's information index (I) were computed for each population based on allele frequencies and calculated for haploid data. In addition, gene diversity within populations (Hs), gene diversity between populations (Dst) by the formula (Dst = Ht − Hs), gene differentiation coefficient (Gst)

Table 1: Description of the 115 sugarcane (*Saccharum* complex) accessions used in the SSR study.

Code	Name of accession	Collection place	Code	Name of accession	Collection place
1	GT86-267	FAFU	59	CP65-357	FAFU
2	GT89-5	FAFU	60	CP67-412	FAFU
3	GT93-103	FAFU	61	CP72-1210	FAFU
4	GT94-116	FAFU	62	CP72-1312	FAFU
5	GT94-119	FAFU	63	CP84-1198	FAFU
6	GT96-44	FAFU	64	CP85-1308	Ruili
7	GT96-211	FAFU	65	*CP88-1762	FAFU
8	GT73-167	Ruili	66	*CP89-1509	FAFU
9	*GT89-7	FAFU	67	*CP92-1167	FAFU
10	*GT90-55	FAFU	68	ROC1	FAFU
11	*GT94-119	FAFU	69	ROC10	Ruili
12	*GT95-53	FAFU	70	ROC11	Ruili
13	*GF97-18	FAFU	71	ROC16	FAFU
14	YT96-835	FAFU	72	ROC20	Ruili
15	YT96-86	FAFU	73	ROC22	Ruili
16	YT00-236	FAFU	74	ROC24	FAFU
17	YT85-633	Ruili	75	ROC25	Ruili
18	YT91-967	Ruili	76	ROC26	FAFU
19	YT93-159	Ruili	77	*ROC2	FAFU
20	YT85-177	Ruili	78	*ROC7	FAFU
21	*YT82-882	FAFU	79	*ROC18	FAFU
22	*YT89-240	Ruili	80	F134	Ruili
23	*YT91-854	FAFU	81	*DZ93-88	Ruili
24	*YT91-1102	FAFU	82	*DZ93-94	Ruili
25	*YT96-244	FAFU	83	*DZ99-36	Ruili
26	*YT97-40	FAFU	84	YZ89-351	FAFU
27	YC71-374	FAFU	85	YZ94-375	Ruili
28	YC82-96	FAFU	86	*YZ92-19	FAFU
29	YC82-108	FAFU	87	*YZ99-91	FAFU
30	YC84-125	FAFU	88	*Q170	FAFU
31	YC89-46	FAFU	89	*Q171	FAFU
32	YC90-3	FAFU	90	*Q182	FAFU
33	YC90-33	FAFU	91	*CZ89-103	Ruili
34	YC92-27	FAFU	92	CZ19	FAFU
35	YC96-48	FAFU	93	*CN85-78	FAFU
36	*YC90-31	FAFU	94	ZZ74-141	FAFU
37	FN91-3623	FAFU	95	ZZ92-126	FAFU
38	FN91-4621	FAFU	96	POJ2878	FAFU
39	FN91-4710	FAFU	97	Co1001	FAFU
40	*FN81-475	FAFU	98	RB72-454	Ruili
41	*FN93-3608	FAFU	99	K5	FAFU
42	*FN94-0744	FAFU	100	GZ8	FAFU
43	*FN02-3924	FAFU	101	LCP85-384	FAFU
44	MT86-05	FAFU	102	*Nagori	Ruili
45	MT86-2121	Ruili	103	*muck che	Ruili
46	MT90-55	FAFU	104	*laica82-1729	Ruili
47	MT92-649	FAFU	105	*YG94-39	FAFU
48	*MT69-421	Ruili	106	*RF93-244	FAFU
49	*MT92-505	FAFU	107	*Brazil45	FAFU
50	*MT93-246	FAFU	108	*FR93-435	FAFU
51	*MT96-6016	FAFU	109	*MEX105	FAFU

Table 1: Continued.

Code	Name of accession	Collection place	Code	Name of accession	Collection place
52	HoCP91-555	FAFU	110	*YZ99-601	FAFU
53	HoCP93-746	Ruili	111	*K16	FAFU
54	HoCP93-750	FAFU	112	*B9	FAFU
55	HoCP95-998	Ruili	113	*PS45	Ruili
56	YN73-204	FAFU	114	*LY97-151	Ruili
57	*YN89-525	FAFU	115	*GN95-108	FAFU
58	CP49-50	FAFU			

Sugarcane Resources Nursery of Fujian Agriculture and Forestry University (FAFU); Ruili Breeding Station in Yunnan Academy of Agriculture Science (Ruili); *represents new parents.

Table 2: The allele detection results of 5 SSR markers used for evaluation of 115 sugarcane accessions.

Primer name	Number of alleles	Number of rare alleles	Range of allele size (bp)	Major allele		PIC
				Size (bp)	Frequency (%)	
SMC334BS	19	6	136–169	147	66.10	0.889
SMC336BS	26	15	136–192	168	59.10	0.897
SMC36BUQ	11	8	101–147	122	39.10	0.753
SMC286CS	15	6	123–169	146	46.10	0.865
SMC569CS	17	11	159–238	220	66.10	0.779
Average	17.6	9.2				0.837

Rare allele means that the frequency of the allele is less than 5.0%; the major allele accounts for the highest proportion in all alleles.

calculated as (Ht − Hs)/Ht, and estimates of gene flow (Nm) were obtained by (1 − Gst)/2Gst. Based on Nei's (1978) genetic distances, a dendrogram showing the genetic relationships between genotypes was constructed by the unweighted pair group method with arithmetic average (UPGMA) using the NTSYS-pc version 2.1 [34, 35]. To further assess the genetic relationships between all of the accessions (9 series), PCA was performed based on genetic similarity using NTSYS-pc version 2.1 [35].

3. Results and Analysis

3.1. SSR Markers. SSR markers were utilized to assess genetic diversity among all the 115 sugarcane parental accessions in this study, and the major values of genetic diversity parameters derived were showed in Table 2.

A total of five SSR loci were used to evaluate 115 sugarcane accessions. Distinct fragments in the size ranging from 101 bp to 238 bp were scored for analysis. The major allele of five SSR loci was observed at the sizes of 147 bp, 168 bp, 122 bp, 146 bp, and 220 bp, with the ratio of 66.1%, 59.1%, 39.1%, 46.1%, and 66.1% with the primers SMC334BS, SMC336BS, SMC36BUQ, SMC286CS, and SMC569CS, respectively. A total of 88 alleles within the data set were obtained, and alleles per locus ranged from 11 to 26, with an average of 17.6. The average number of rare alleles produced in a single individual was 9.2 (range 6–15). The highest number of alleles was scored at locus SMC336BS (26 alleles). The PIC values of five SSR loci ranged from 0.753 to 0.897 with a mean value of 0.837. The PIC value of the SMC336BS locus was the highest (0.897), while the lowest (0.753) was observed from SMC36BUQ locus.

3.2. Genetic Diversity among 64 Common Parents, 51 New Parents, and All 115 Parents. Significant genetic variation was found among all 115 parents with the genetic similarity (GS) value ranging from 0.725 to 1.000. The GS value ranged from 0.730 to 1.000 within the group of 64 common parents and from 0.722 to 0.943 within the group of 51 new parents. Of note, the GS value was 1.000 between MT90-55 and HoCP93-750, indicating that there was no genetic dissimilarity between the two parents based on the five SSR loci.

Genetic parameters for the five microsatellite loci in the two groups, common parents and new parents, were given in Table 3. A total of 88 polymorphic bands within the entire data set were scored, while taking the two groups considered separately, 82 of them were within the 64 common parents (93.18%), and 69 of them were within the 51 new parents (78.41%). Observed numbers of alleles (Na) were the same (2.000) in the two groups, and effective numbers of alleles (Ne) were higher in new parents group (1.359) than in common parents group (1.302). Nei's gene diversity (h) was 0.178, and Shannon's information index (I) was 0.288 in the overall sugarcane testing accessions. In contrast to the total diversity, both sugarcane parent groups of common parents and new parents had relatively high diversity, h = 0.190 and 0.223 and I = 0.308 and 0.356, respectively.

Table 4 summarized the genetic differentiation of sugarcane accessions from the two groups. The values of Ht and Dst were higher in new parents group (Ht = 0.214, Dst = 0.058) than those in common parents group (Ht = 0.190, Dst = 0.032), while the value of genetic diversity (Hs) within population was similar in two groups (0.158 for common

Table 3: The values of genetic diversity parameters for sugarcane accessions of common and new parents in different groups, estimated based on polymorphisms of 5 SSR loci.

Group	Clones size	NPB	PPB (%)	Na	Ne	h	I
Common parents	64	82	93.18	2.000	1.302	0.190	0.308
New parents	51	69	78.41	2.000	1.359	0.223	0.356
Total	115	88	100.0	2.000	1.283	0.178	0.288

Number of polymorphic bands (NPB); percentage of polymorphic bands (PPB); observed number of alleles (Na); effective number of alleles (Ne); Nei's genetic diversity (h); Shannon's information index (I).

Table 4: Genetic diversity and differentiation of sugarcane accessions between common and new parents, estimated by POPGENE (version 1.31).

Group	Clones size	Ht	Hs	Dst	Gst	Nm
Common parents	64	0.190	0.158	0.032	0.171	2.429
New parents	51	0.214	0.156	0.058	0.273	1.335
Total	115	0.176	0.159	0.017	0.095	4.762

Mean values of total gene diversity (Ht), gene diversity within populations (Hs), gene diversity between populations (Dst), gene differentiation coefficient (Gst), and estimates of gene flow from Gst (Nm) were obtained by (1 − Gst)/2Gst.

Table 5: Genetic diversity of sugarcane parents in 9 series released by different breeding institutions, estimated based on polymorphisms of 5 SSR loci.

Series	Clones size	NPB	PPB (%)	Na	Ne	h	I
GT	13	47	53.41	1.534	1.262	0.162	0.250
YT	13	50	56.82	1.568	1.267	0.166	0.258
YC	10	49	55.68	1.557	1.283	0.178	0.275
FN	7	37	42.05	1.421	1.236	0.144	0.219
MT	8	44	50.00	1.500	1.259	0.161	0.247
HoCP	4	32	36.36	1.364	1.268	0.152	0.221
CP	10	36	40.91	1.409	1.222	0.136	0.181
ROC	13	46	52.27	1.523	1.259	0.160	0.247
OTHER	37	62	70.45	1.705	1.290	0.177	0.278

parents group and 0.156 for new parents group), indicating that the genetic diversity of these two groups mainly existed within populations. The gene flow index (Nm) within groups showed that low gene flow (2.429 and 1.335, resp.) occurred in both groups, while the Gst was high in both groups—0.171 and 0.273, respectively. The gene flow between the two groups was much higher (Nm = 4.762) than those in both groups. This also indicated that the genetic variation mainly existed within populations.

3.3. Genetic Relationships of 115 Sugarcane Parents. Nine series from 115 accessions sorted by institution-based breeding programme are shown in Table 5. According to the information indicated in Table 1, we assigned them as the following nine series: GT series (13) from Guangxi Sugarcane Institute; YT series (13) from Guangzhou Institute of Sugarcane and Sugar Industry; YC series (10) from Hainan Sugarcane Breeding Station; FN series (7) from Sugarcane Research Institute of FAFU; MT series (7) from Sugarcane Research Institute, Fujian Academy of Agricultural Sciences; HoCP series (4) from Sugarcane Research Unit, Houma, Louisiana, United States Department of Agriculture, USA; CP series (10) from Sugarcane Experiment Station, Canal Point, Florida, United States Department of Agriculture, USA; and "ROC" series (13) from Taiwan Sugar Corporation. The rest of the sugarcane parents included 37 accessions from several breeding institutions different from all the above eight and were termed as OTHER.

Genetic diversity parameters for the 5 microsatellite markers in the 9 sugarcane series were presented in Table 5, indicating that except the highest NPB value (62 stands for 70.45%) observed in OTHER series, the polymorphisms among eight determinate series were as follows: YT (50, 56.82%) > YC (49, 55.68%) > GT (47, 53.41%) > "ROC" (46, 52.27%) > MT (44, 50.00%) > FN (37, 42.05%) > CP (36, 40.91%) > HoCP (32, 36.36%). Observed numbers of alleles (Na) were higher in OTHER (Na = 1.705) and YT (Na = 1.568) series compared to those of the remaining seven series. Moreover, effective numbers of alleles (Ne) were also higher in OTHER (Ne = 1.290) and YC (Ne = 1.290) series compared to those of the remaining seven determinate series. Except OTHER series ($h = 0.177$, $I = 0.278$), both the gene diversity (h) and the Shannon information index (I) were higher in YC ($h = 0.178$, $I = 0.275$) series but lower in CP series ($h = 0.136$, $I = 0.181$).

The number of alleles based on 5 SSR loci in different series of GT, YT, YC, FN, MT, HoCP, CP, "ROC," and OTHER was illustrated in Figure 1. A total of 1,395 alleles

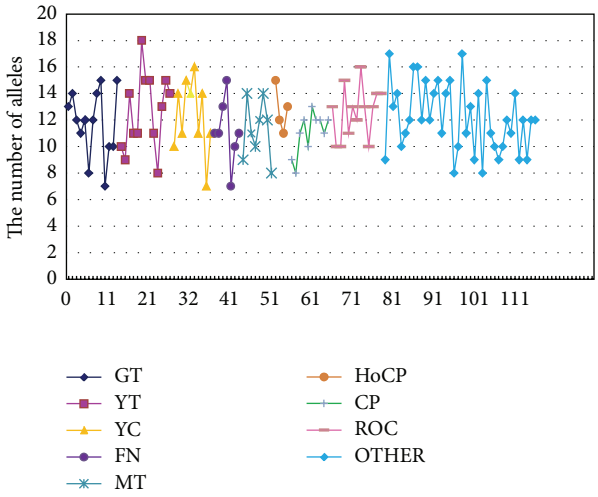

Figure 1: The number of alleles detected in 115 sugarcane accessions based on 5 SSR loci.

were detected for all the 115 testing sugarcane accessions with an average of 12. The maximum number of alleles was 18 observed in YT93-159, while the minimum number was 7 in three accessions of GT90-55, YC96-48, and FN93-3608. Within the GT and FN series, the number of alleles both ranged from 7 to 15 with mean values of 11.8 and 11.1, respectively. In YT series, the number of alleles per locus ranged from 8 to 18 with an average of 12.6. Within MT series, the number of alleles ranged from 8 to 14, and the average number was 11.3. In HoCP series, the number of alleles was located between 11 and 15 with an average of 12.8. Within CP series, the number of alleles ranged from 8 to 13 with an average of 11.0. In "ROC" series, the number of alleles ranged from 10 to 16 with an average of 12.5. Within OTHER series, with an average of 12.0, the number of alleles was from 8 to 17.

3.4. Cluster Analysis. The measure of genetic distance (GD) can be applied to any kind of organism without regard to ploidy or mating scheme [36], with genetic distance estimates hardly affected by the sample size [37]. Therefore, in this study, a UPGMA dendrogram was constructed based on Nei's genetic distance (Figure 2), showing the genetic relationships among the various series, including single series of GT, YT, YC, FN, MT, HoCP, CP, and "ROC" and complex series of OTHER and that between two groups of common parents (64) and new parents (51). The 115 sugarcane parents were classified into three groups (Group I, Group II, and Group III) at the level of GD = 0.03. Group I consisted of 53 common parents and 38 new parents, including 10 from GT, 12 from YT, 7 from YC, 5 from FN, 6 from MT, 4 from HoCP, 9 from CP, 11 from "ROC," and 27 from OTHER. Group II contained 3 common parents and 4 new parents, including 1 from GT, 2 from YC, 1 from CP, and 3 from OTHER. Group III contained 8 common parents and 9 new parents, including 2 from GT, 1 from YT, 1 from YC, 2 from FN, 2 from MT, 2 from "ROC," and 7 from OTHER. At the level of GD = 0.09, Group I could be further divided into five subgroups (Subgroup Ia, Ib, Ic, Id, and Ie). Ia contained 15 common parents and 8 new parents, including 3 from GT, 2 from YT, 3 from YC, 2 from FN, 3 from MT, 3 from CP, and 7 from OTHER. Ib consisted of 30 common parents and 27 new parents, including 5 from GT, 10 from YT, 4 from YC, 3 from FN, 3 from MT, 2 from HoCP, 4 from CP, 10 from "ROC," and 16 from OTHER. Ic had only two parents from YT containing 1 common parent and 1 new parent. Id contained 3 parents from each of HoCP, CP, and "ROC" and belonged to common parents. Ie contained 4 common parents and 2 new parents, including 1 from HoCP, 1 from CP and 4 from OTHER series.

It should be noted that Group I included most of the parents which came from different series. The above results demonstrate that the genotypes released by the same breeding institutions showed closer genetic relationships, except YC series released by Hainan sugarcane breeding station, which aimed at sugarcane germplasm innovation. It suggested that these parents should be useful in sugarcane cross breeding due to various genetic distances among them. Besides, a total of four testing accessions, including pairs of YT96-86 and YN73-204, plus MT90-55 and HoCP93-750, could not be distinguish based on the 5 microsatellite markers, and it may be due to their sharing of similar basis of genetic background.

3.5. Principal Component Analysis. PCA examined a dissimilarity matrix of pairwise differences between specimens and used eigenvalue analysis in order to take the variation between specimens and condense them into a limited number of dimensions. The maximum amount of variation was plotted as the first axis, with subsequent variation of lesser magnitude explained by each additional dimension [38]. The principal component analysis, which can be helpful for illustrating the genetic relationships of sugarcane parents as individual units, was calculated based on the SSR data matrix of the 5 loci for all 115 sugarcane accessions occupied in this study (Figure 3). The first and second principal components accounted for a cumulative 76% of the variance, including 43% for common parents and 33% for new parents, respectively. As shown in Figure 3, 115 sugarcane parents were scattered in a limited space, covering 90% of CP series, 85% of YT and "ROC" series, 77% of GT series, 75% of MT and HoCP series, 73% of OTHER series, 71% of FN series, and 50% of YC series, respectively. We found that the distribution of sugarcane accessions in CP, YT, and "ROC" series was relatively narrow, while it was wider in YC, FN, and OTHER series. This revealed that genetic basis of the latter group was more extensive than the former group. Furthermore, the plots of two pairs of sugarcane accessions (YT96-86/YN73-204 and MT90-55/HoCP93-750) overlapped strongly (Figure 3). This analysis could not differentiate YT96-86 from YN73-204 or MT90-55 from HoCP93-750 at least at a molecular level based on the 5 SSR markers used in this study.

4. Discussion and Conclusions

Improvement of sugarcane by genetic manipulation has been ongoing since 1888, following the observation in 1858 that sugarcane produced viable seed [1, 39]. According to the studies of Chen et al. (2011) and of Baver (1963), the

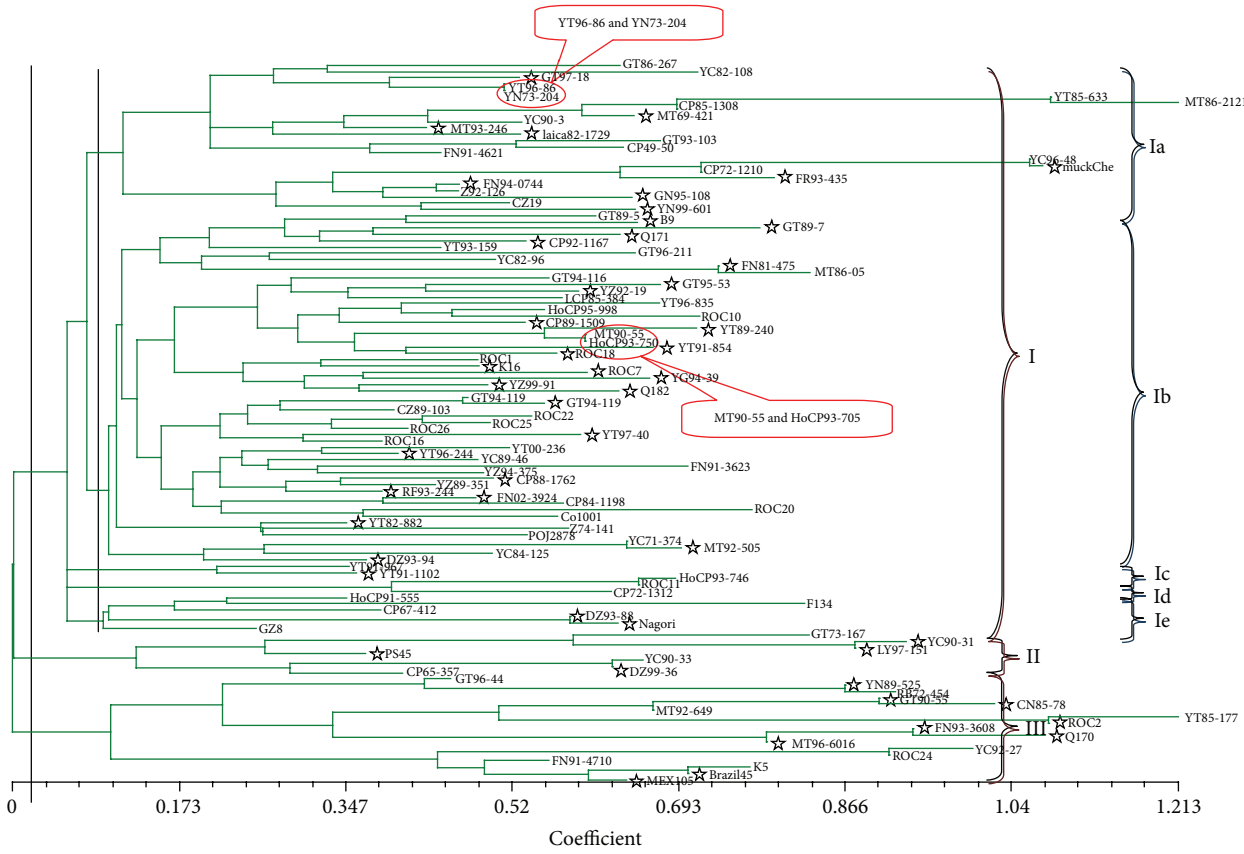

Figure 2: The UPGMA dendrogram of 115 Sugarcane parents based on 5 pairs of SSR primers.

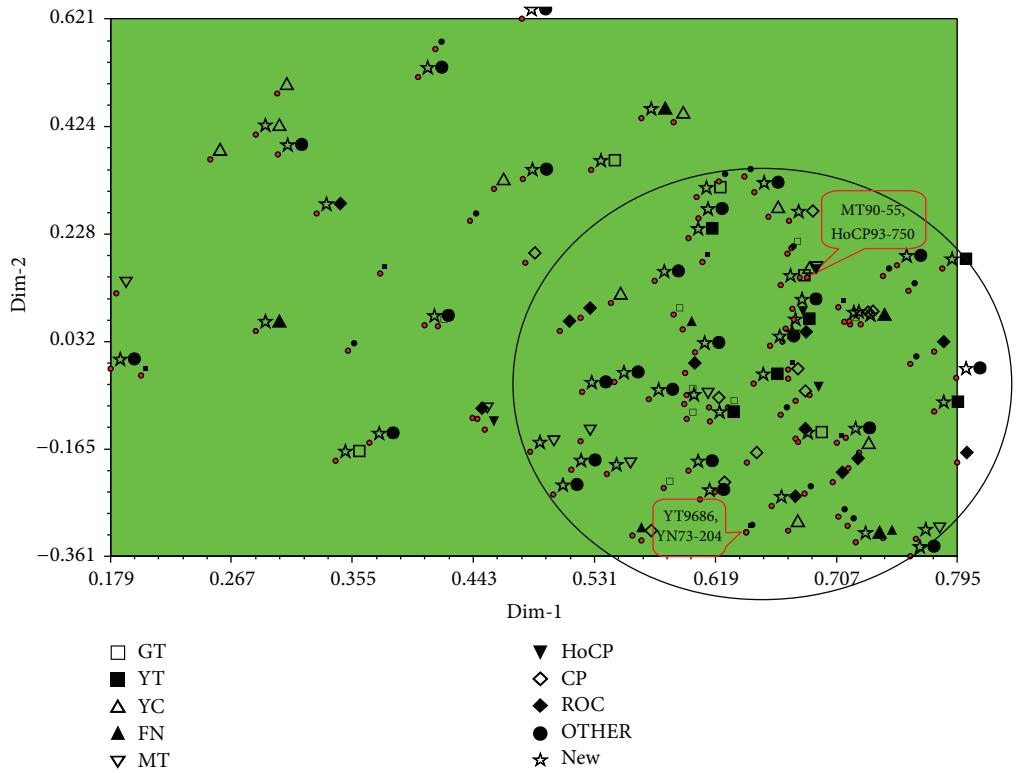

Figure 3: Principal coordinates analysis (PCA) of 115 sugarcane parents using 5 pairs of SSR markers based on genetic similarity.

contribution based on genetic improvement to increase the yield of cane sugar was estimated to be 75% of the yield increase attained by the Hawaiian sugar industry in the 1950s and more than 60% in the Chinese sugar industry in the last three decades [1, 40]. In Hawaii, the yield has improved every decade except in the 1970s, when disease problems plagued the sugar industry [40]. Although the degree to which varietal improvement has contributed to increase yield potential has varied widely from nation to nation, undoubtedly all nations have benefited to some degree by converting to newer, improved varieties from cross breeding. In addition, sugarcane is a potential bioenergy crop due to its high yield and high biomass. The world record and average in Hawaii (1978–1982) are 24.2 and 11.9 metric tons/ha/year, respectively. The 11.9 metric tons/ha/year represents a sugarcane dry matter yield of only 0.07 mt/ha/day, which is much lower than the theoretical maximum of 0.7 mt/ha/day estimated by Loomis and Williams [41].

In China, approximately 400 sugarcane varieties have been released in the last 50 years by cross breeding [42]. However, most of the sugarcane cultivars in the world can be dated back to only a few common ancestors [1, 19]. This may be due to the problem that the genetic basis of the sugarcane is limited; thus, new cultivars with interesting traits are difficult to be developed [43]. A similar situation has occurred in China, where the major cultivars in the 1980s, 1990s, and 2000s were ROC10, ROC16, and ROC22, respectively. Thus, till now, the heterogeneity of cultivars has been very low since the variety ROC22 takes about 50%–60% of the total sugarcane planting area. This limits any further increase of sugar yield per unit and has many potential risks of suffering from common diseases [1]. Sugarcane cross breeding largely depends on broadening the genetic basis and the selection of parents for crossing. The Hainan Sugarcane breeding station is responsible for sugarcane hybridization in China, innovation targets of parents, and introduction of new parents into sugarcane hybridization programs. An increase in the genetic diversity of parental accessions should be helpful to broaden the genetic basis of the sugarcane [26, 44].

In the present study, the genetic diversity of 115 sugarcane parents was evaluated based on 5 microsatellite loci. These SSR markers were highly robust and codominant as characterized by high PIC value (0.84 on average), but exhibited the lower level of polymorphism described by Liu (2011) who reported average PIC value = 0.70 [24]. However, the level of polymorphism obtained in our and Pan's studies was much higher than other SSR markers reported by Filho et al. (2010), who reported mean PIC value = 0.57 [45]. Genetic diversity of different series including eight determinate and one complex (OTHER) series showed that YC series had higher genetic diversity ($h = 0.188$ and $I = 0.275$) except OTHER ($h = 0.177$ and $I = 0.278$) and that CP and FN series had lower ones ($h = 0.136$ and 0.144, $I = 0.181$ and 0.219, resp.). This is consistent with the results reported by Li et al. (2005) and Lao et al. (2008) [46, 47].

In the present study, all 64 accessions in common parents group showed relatively lower diversity, compared with the higher diversity exhibited by 51 accessions of new parents group. The result was based on the value of Nei's genetic diversity ($h = 0.190 < 0.223$) and Shannon's information index ($I = 0.308 < 0.356$), indicating that the innovation of parents has showed a positive role in sugarcane breeding programs in China, since the group of new parents has higher genetic diversity, and thus, it will to some degree benefit the broadening of the genetic basis in sugarcane hybridization.

The values of Nei's genetic diversity and Shannon's information index were much lower in other series than those in two groups. However, the level of diversity obtained in our research (two groups) was similar to previous research, which reported Nei's genetic diversity $h = 0.222$ and Shannon's information index $I = 0.328$ [13]. Since gene flow can resist the effect of genetic drift within populations and prevent the differentiation of populations with $Nm > l$, the genetic drift would lead to genetic differentiation among populations as the value of $Nm < l$ [48]. The Nm value in this study was 4.762, indicating that there was no significant genetic differentiation between the two groups or nine series. The low genetic differentiation (Gst) among populations was primarily caused by the high level of gene flow. However, compared to wild sugarcane (Gst = 0.209) [13] and weedy rice (Gst = 0.387) [38], the Gst (0.095) of 115 sugarcane parents was still at a low level.

It is interesting that, in this study, both cluster and PCA analyses of individuals (including all the nine series) exhibited similar results: OTHER, YC, and GT series fell into three different groups and HoCP only belonged to Group I. Furthermore, a limited space covered 90% CP series, 85% YT and "ROC" series, and only 50% YC series, respectively. It was obvious that the distribution of accessions in CP, YT, and "ROC" series was relatively narrow while it was broader in YC, FN, and OTHER series. The results revealed that the genetic basis of YC, FN, and OTHER was more extensive than CP, YT, and "ROC" series, which also suggested that more attention should be made on the application of new parents in sugarcane hybrid breeding in the future. It was not difficult to find in the dendrogram (Figure 2) and PCA (Figure 3) that the clusters or components were closely related to their breeding institutions.

It was also apparent that there were two pairs of four accessions (YT96-86 and YN73-204 at the level of GD = 0.50 and MT90-55 and HoCP93-750 at the level of 0.59) which the analysis failed to differentiate. Furthermore, the PCA analysis indicated that the plots of YT96-86 and YN73-204 or MT90-55 and HoCP93-750 overlapped entirely. This shows that the analysis could not differentiate between these accessions at the molecular level based on the five testing SSR loci and indicated that more SSR loci would be necessary for differentiation from MT90-55 to HoCP93-750 and from YT96-86 to YN73-204. For example, based on the pedigree, HoCP93-750 evolved from CP84-0722 and LCP81-030, while MT90-55 derived from CP57-614 and YC84-153 (Figures 4 and 5). From the pedigree of HoCP93-750 and MT90-55, it is obvious that we could not find the same parents between the two sugarcane clones within five generations. Therefore, it is inaccurate to analyze the genetic structures, genetic diversity, or genetic relationships only by pedigree records. If we want

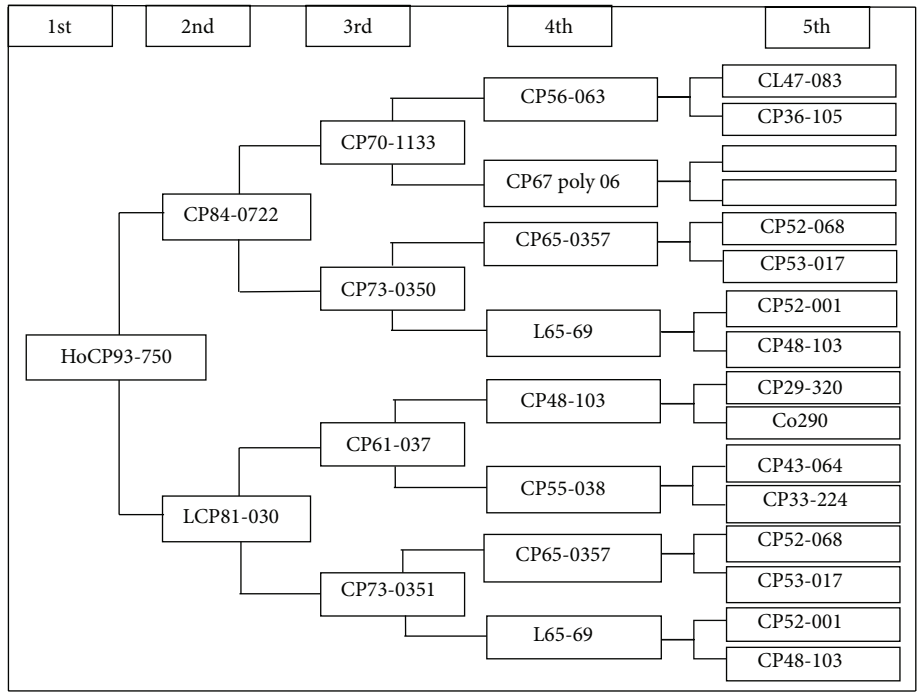

Figure 4: The pedigree of HoCP93-750.

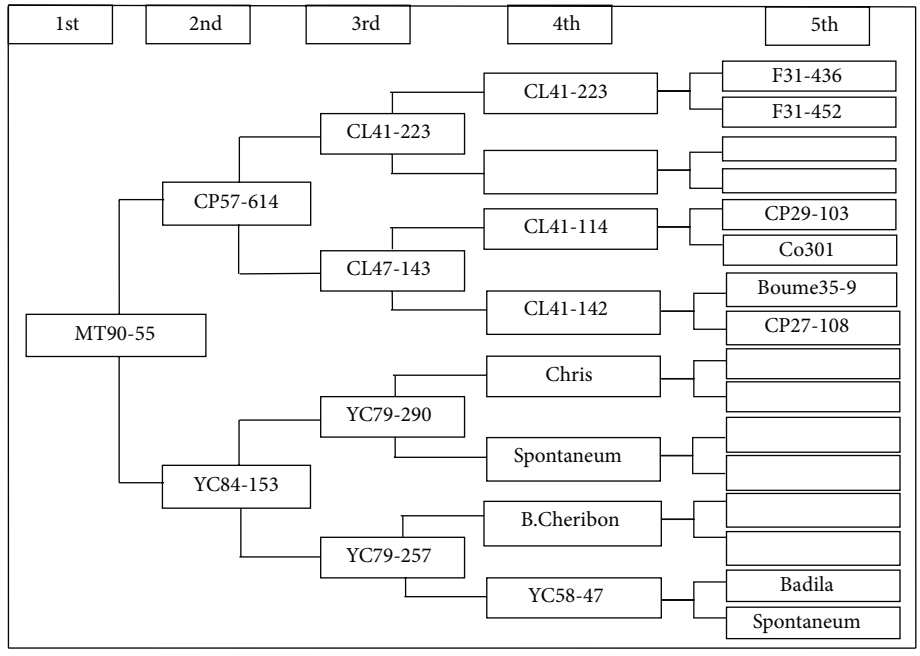

Figure 5: The pedigree of MT90-55.

to further identify the four sugarcane clones, more SSR loci should be applied.

According to previous reports, gSSR markers produce polymorphisms based on the difference in the number of DNA repeat units in regions of the genome and derive from genomic DNA libraries at a high price, while EST-SSRs detect variations in the expressed portion of the genome and can be mined from the EST databases at low price [20, 49, 50]. EST-SSR technology has been widely used in many plants, such as rice [51], sorghum [52], wheat [53], and several other plant species. However, the usefulness of EST-SSRs varies in different varieties of sugarcane, as the level of polymorphism (PIC = 0.23) was lower than that of anonymous SSR markers (PIC = 0.72) in sugarcane cultivars. It was also reported that

EST-SSRs had higher level of polymorphism across ancestral species (PIC = 0.66 > 0.62) [20]. In other research, the number of alleles of gSSRs loci (7–9) was more than EST-SSRs loci (4–6), and about 35% of the gSSRs had PIC values around 0.90 in contrast to 15% of the EST-SSRs (50). What should also be stressed is that the two types of SSR, gSSR and EST-SSR, made no significant difference at the average genetic similarity (GS) based on Dice coefficient and were in good agreement with pedigree information for genetic relationships analysis [50]. These results demonstrated that, in the future, EST-SSRs should be used together with gSSRs for genetic relationship analysis in sugarcane.

From the above discussion, identifying useful gSSRs is significant, but in sugarcane, this can be a lengthy and difficult process due to their complexity and their abundance within the sugarcane genome [20, 50, 54]. Therefore, there is further work required to promote this technique. This paper used only 5 pairs of gSSR primers in the genetic diversity analysis of 115 sugarcane parents in spite of the testing SSR loci being selected from a batch of gSSR loci (221 ICSB sugarcane SSR markers) and having shown to be robust and polymorphic. This suggests that more basic *Saccharum* species, more gSSR markers, and more molecular methods like EST-SSRs can be utilized in further study.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Genetic Transformation in *Citrus*

Dicle Donmez,[1] Ozhan Simsek,[2] Tolga Izgu,[3] Yildiz Aka Kacar,[1,2] and Yesim Yalcin Mendi[1,2]

[1] *Biotechnology Department, Institute of Applied and Natural Sciences, Çukurova University, 01330 Adana, Turkey*
[2] *Horticulture Department, Agriculture Faculty, Çukurova University, 01330 Adana, Turkey*
[3] *Horticulture Department, Agriculture Faculty, Ege University, 35100 İzmir, Turkey*

Correspondence should be addressed to Yildiz Aka Kacar; ykacar@cu.edu.tr

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Citrus is one of the world's important fruit crops. Recently, citrus molecular genetics and biotechnology work have been accelerated in the world. Genetic transformation, a biotechnological tool, allows the release of improved cultivars with desirable characteristics in a shorter period of time and therefore may be useful in citrus breeding programs. *Citrus* transformation has now been achieved in a number of laboratories by various methods. *Agrobacterium tumefaciens* is used mainly in citrus transformation studies. Particle bombardment, electroporation, *A. rhizogenes*, and a new method called RNA interference are used in citrus transformation studies in addition to *A. tumefaciens*. In this review, we illustrate how different gene transformation methods can be employed in different citrus species.

1. Introduction

Citrus species are the most widely grown fruit crops. Despite substantial genetic diversity and interspecific fertility, the genus *Citrus* includes some of the most difficult species to breed [1, 2]. This is due to several obstacles for conventional breeding. For example, most species are highly heterozygous and produce progeny that segregate widely for many characters when crosses are made. The juvenile periods are often very long, self- and cross-incompatibility and pollen and/or ovule sterility are relatively common, and the presence of adventitious somatic embryos in the nucellus of developing ovules of the most of *Citrus* greatly limits hybrid production [2, 3].

The genus *Citrus* possesses several undesirable characteristics including salt and cold sensitivity [4, 5]; they are also susceptible to diseases caused by fungi, bacteria and viruses, such as *Citrus* exocortis viroid (CEV), *Citrus* infectious variegation virus (CIVV), *Citrus* cachexia viroid (CCaV) and *Citrus* tristeza closterovirus (CTV) [5, 6]. Classical genetic selection, gene transfer, grafting, and micrografting techniques can contribute to the improvement of *Citrus* and propagation of selected species. Therefore, *in vitro* manipulation procedures leading to a rapid, direct bud regeneration for efficient micropropagation as well as genetic transformation are needed as a first step towards *Citrus* improvement. Practical benefits resulting from *in vitro* culture methods have already been reported in *Citrus* [5, 7, 8]. Recent developments in gene transfer techniques via the classical regeneration method have been applied to this genus and have opened the way to induce a specific genetic change within a period of time shorter than using the classical genetic selection method [5, 9, 10].

Conventional breeding methods have demonstrated limitations with respect to citrus improvement due to some of the biological characteristics of woody plants such as nucellar polyembryony, high heterozygosity, long juvenile period, and autoincompatibility [11, 12]. The development of biotechnological tools has made it possible to overcome some of these problems. In the specific case of citrus breeding programs, somatic hybridization [12–14] and genetic transformation [12, 15, 16] have been applied in many countries [10, 12, 17, 18].

In recent years, there has been a major thrust in citrus improvement as competition from international citrus markets, disease, and pest pressure and other abiotic and biotic stress conditions stimulate worldwide interest [19, 20]. Several strategies exist for the genetic improvement of citrus including conventional breeding and genetic transformation [20, 21]. Currently, genetic transformation of citrus as a tool

for citrus improvement is gaining popularity. This method is especially useful in cases where it is not possible to introduce a particular trait of interest to another elite cultivar using conventional breeding. *Citrus* cultivars vary in their response to *in vitro* organogenesis and genetic transformation. This results in the need for cultivar-specific optimization of *in vitro* protocols [20, 22].

Among the several methods available for the genetic transformation of citrus, the most popular method to transform a wide range of citrus cultivars is *Agrobacterium*-mediated transformation using epicotyl explants as target cells for incorporation of the T-DNA [20, 23]. However, this method is not suitable for the transformation of any seedless cultivar. Also, special cultivars in the mandarin group remain robust to transform using this method [20, 22, 24].

2. Transformation Studies in *Citrus*

Genetic transformation and somatic hybridization studies are already integrated in *Citrus* breeding programs in several countries. Genetic transformation of *Citrus* is a promising tool that enables the introduction of desirable traits without altering the genetic background [25]. Genetic transformation of citrus has been reported, by using several methods (Table 1).

Agrobacterium has been the most frequently used genetic transformation method in *Citrus* with explants collected from seedlings germinated *in vitro* or under greenhouse conditions [68].

Transformation studies have been done for two decades in citrus. In the last few years, different transformation methods such as RNA silencing are used. In order to carry out successful gene transformation studies in citrus, optimized *in vitro* regeneration protocol is needed. Researchers should optimize efficient regeneration protocol before starting transformation studies. There are also many efficient regeneration protocols published in different citrus species.

Orbović et al. [36] investigated the effects of seed age on shoot regeneration potential and transformation rate of "Duncan" and "Flame" grapefruit cultivars, along with "Hamlin" sweet orange cultivar. Genetic transformation of citrus explants was carried out as previously described [93] using *A. tumefaciens* strain EHA105 [94] containing a binary vectors derived from pD35s [22]. In conclusion, the regeneration potential and transformability of citrus juvenile explants are different among cultivars and also change within the fruit harvest season. Because of these findings, especially the latter one, it will be extremely difficult to develop a universal protocol for genetic transformation of citrus. Optimal transformation efficiency will require flexible procedures that account for cultivar variability and timing of seed collection. In another study, a protocol was developed for regeneration of transgenic plants via *A. tumefaciens*-mediated transformation of leaf segments from "Valencia" sweet orange (*C. sinensis* L. Osbeck) using *gfp* (green fluorescence protein) as a vital marker [27]. The transformation methodology described by Khan et al. [27] was an important finding for generating transgenic plants using leaf segments as explants.

In addition to transformation studies via *A. tumefaciens*, recently, *A. rhizogenes* has been used. Many reports suggest the use of *A. rhizogenes* for expression of the rol genes and also to deliver foreign genes to susceptible plants [95]. The hairy root harbours the T-DNA segment of Ri-plasmid within its nuclear genomes [96]. *A. rhizogenes* are also capable of transferring the T-DNA of binary vectors *in trans*, thereby facilitating the selection of transgenic plants from screened hairy roots [95]. *A. rhizogenes*-mediated transformation system was found to be very useful in genetic manipulation of plants for the production of phytochemicals [97], large scale secondary metabolite production [98], monoclonal antibody production [99], and phytoremediation [100]. There are many reports that suggest the successful use of *A. rhizogenes* harbouring binary vectors with desired gene constructs [95] for plant genetic transformation [101]. Due to low transformation efficiency of *A. rhizogenes*, many researchers have worked to optimize transformation methods.

Chávez-Vela et al. [72] used *A. rhizogenes* A4 agropine-type strain to develop the transformation system. A4 contains wild-type plasmid pRi A4 which confers hairy-root genotype and binary vector pESC4. In the study seventy-five-day-old sour orange seedlings were used and transgenic sour orange (*C. aurantium* L.) plants were regenerated from *A. rhizogenes* transformed roots. 91% of explants produced transformed roots with an average of 3.6 roots per explant.

In another study transgenic Mexican lime (*C. aurantifolia* (Christm.) Swing) plants were regenerated from tissues transformed by *A. rhizogenes* strain A4, containing the wild-type plasmid pRiA4 and the binary vector pESC4 with nos-npt II and cab-gus genes. More than 300 Mexican lime transgenic plants were obtained, 60 of which were adapted to growing in soil [2].

In addition to the indirect gene transfer methods, there are studies performed by direct gene transfer methods in citrus. Bespalhok Filho et al. [69] carried out to optimize the conditions for transient gene expression through particle bombardment on Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) thin epicotyl sections. The best conditions for transient GUS expression were M-25 tungsten particles, 1550 psi helium pressure, 9 cm distance between specimen, and DNA/particle holder and culture of explants in a high osmolarity medium (0.2 M mannitol + 0.2 M sorbitol) 4 h prior and 20 h after bombardment. Under these conditions, an average of 102 blue spots per bombardment (20 explants/plate) were achieved. It is stated that protocol is currently being used for transformation of Carrizo citrange and sweet orange (*C. sinensis*).

Electroporation is an effective direct gene transfer system used for citrus transformation. Hidaka and Omura [90] used electroporation methods for gene transformation in citrus. Protoplasts were prepared from embryogenic callus of "Ohta" ponkan (*C. reticulata* Blanco) and electroporation with exponential decay pulses was carried out in the solution containing the β-glucuronidase (GUS) chimeric gene coupled to the CaMV 35S promoter (pBI221). At 24 hr after incubation, significant GUS activity was detected in the cells by fluorometric assay. Another alternative method for direct gene transformation had been developed in sweet

Table 1: Transformation researches in citrus.

Species	Transferred genes	Transformation method	References
C. sinensis L. Osb.	*GUS* and *npt*II	*A. tumefaciens*	[26]
C. sinensis L. Osb.	*gfp*	*A. tumefaciens*	[27]
C. sinensis L. Osb.	*GUS*	*A. tumefaciens*	[28]
C. sinensis L. Osb. and Carrizo citrange	*uid*A, *npt*II	*A. tumefaciens*	[29]
C. paradisi Macf.	*RdRp*, *Gfp*, and *Gus*	*A. tumefaciens*	[30]
C. sinensis L. Osb.	*CTV-CP*	*A. tumefaciens*	[31]
C. aurantifolia	*p25*, *p20*, and *p23*	RNA interference	[32]
C. aurantifolia Swingle	*AtSUC2, RSs*1, *RTBV, GUS*, *rol*C	*A. tumefaciens*	[33]
C. paradisi	*att*E	*A. tumefaciens*	[34]
C. unshiu Marc	*miraculin*	*A. tumefaciens*	[35]
C. sinensis L. Osbeck and *C. paradisi* Macf.	*GFP*	*A. tumefaciens*	[36]
C. sinensis L.	*CTV-GFP*	*A. tumefaciens*	[37]
C. sinensis Osb.	*Shiva* A and *Cecropin* B	*A. tumefaciens*	[38]
C. sinensis	CPsV *cp* (ihpCP), *54K* (ihp54K), and *24K* (ihp24K)	*A. tumefaciens* RNA silencing	[39]
C. sinensis L. Osb.	*GFP* and *npt*II	*A. tumefaciens*	[40]
C. sinensis L. Osb.	*pth*A-*nls*	*A. tumefaciens*	[41]
Poncirus trifoliata L. Raf.	*AhBADH*	*A. tumefaciens*	[42]
C. sinensis L. Osb.	*Cy-GFP* and *Er-GFP*	*A. tumefaciens*	[43]
C. aurantifolia Swingle	*gus-egfp*	*A. tumefaciens*	[44]
Tetraploid citrus rootstock selection "Orange #16"	*egfp-npt*II	*A. tumefaciens*	[45]
Carrizo citrange	*man*A and *egfp*	*A. tumefaciens*	[22]
C. sinensis, *C. reticulata C. amblycarpa* and *C. depressa*	*npt*II, *hpt*II, *and GFP*	*A. tumefaciens*	[20]
Carrizo citrange and *C. sinensis* L. Osb.	*Gfp*	*A. tumefaciens*	[46]
Carrizo citrange, *C. paradisi* Macf., *C. aurantifolia* Swingle *C. sinensis* L. Osb.	*EGFP*	*A. tumefaciens*	[23]
"Swingle" citrumelo and *C. sinensis* L. Osb.	*GUS* and *npt*II	Sonication-assisted *A. tumefaciens* (SAAT)	[47]
C. sinensis cv. Hamlin	*hrp*N	*A. tumefaciens*	[48]
Poncirus trifoliata [L.] Raf.	*uid*A and *npt*II	*A. tumefaciens*	[49]
Poncirus trifoliata [L.] Raf	*GFP* and *MAC12.2*	*A. tumefaciens*	[50]
Carrizo citrange and *C. sinensis* L. Osb.	*uid*A and iaaM/H marker genes	*A. tumefaciens*	[51]
C. sinensis L. Osb.	*cp* and *nos* genes	*A. tumefaciens*	[52]
C. sinensis L. Osb.	Nospro-nptII-Noster	*A. tumefaciens*	[53]
Carrizo citrange and *C. sinensis* L. Osb.	*ipt* gene	*A. tumefaciens*	[54]
C. paradise Macf.	*CTV*-derived candidate resistance	*A. tumefaciens* RNA-mediated resistance	[55]
Poncirus trifoliata L. Raf.	*gfp*	*A. tumefaciens*	[56]
Carrizo citrange	*npt*II	*A. tumefaciens*	[57]
C. aurantium, C. macrophylla, C. limon and Troyer citrange	*CTV-p61* and *p23U*	*A. tumefaciens*	[58]
C. sinensis L. Osb.	*att*A	*A. tumefaciens*	[59]
C. limonia Osb.	*bO*	*A. tumefaciens*	[60]
C. paradisi	*RdRp*	*A. tumefaciens*	[61]

Table 1: Continued.

Species	Transferred genes	Transformation method	References
C. jambhiri Lush	*GUS* and *npt*II	*A. tumefaciens*	[62]
C. sinensis L. Osb.	*gfp* and *pme*	PEG	[63]
Swingle citrumelo	*uid*A, *npt*II, and *GUS*	*A. tumefaciens*	[64]
Carrizo citrange	*GUS* and *npt*II	*A. tumefaciens*	[65]
Carrizo citrange and *C. aurantifolia*	*GUS, GFP*, and *npt*II	*A. tumefaciens*	[66]
Carrizo citrange	Citrus blight-associated	*A. tumefaciens*	[67]
C. sinensis and *C. limonia*	*GUS*	*A. tumefaciens*	[68]
Carrizo citrange	*uid*A and *npt*II	Particle bombardment	[69]
C. sinensis	*pTA29-barnase*	*A. tumefaciens*	[70]
Citrus sinensis	*PMI*	*A. tumefaciens*	[12]
Citrus sinensis	*GUS* and *npt*II	*A. tumefaciens*	[71]
Citrus aurantium L.	*GUS* and *npt*II	*A. rhizogenes*	[72]
Citrus paradisi Macf.	*cp* and GUS	*A. tumefaciens*	[73]
C. sinensis L. Osb.	*GUS*	Electroporation	[74]
Carrizo citrange and *C. sinensis* L. Osb.	*GUS*	*A. tumefaciens*	[75]
Citrus sinensis L. Osbeck	*GUS* and *npt*II	*A. tumefaciens*	[18]
C. reticulata Blanco	*pTA29-barnase*	*A. tumefaciens*	[76]
C. paradisi Macf.	Carotenoid biosynthetic genes	*A. tumefaciens*	[16]
Carrizo citrange	*LFY* and *API*	*A. tumefaciens*	[77]
C. aurantium L.	*cp*	*A. tumefaciens*	[78]
C. paradisi Macf.	*CP* and *T36*	*A. tumefaciens*	[79]
Troyer citrange	*Bar* and *uid*A	*A. tumefaciens*	[80]
C. aurantifolia Swing.	*cp*	*A. tumefaciens*	[81]
C. sinensis (L.) Osb.	*Gfp*	PEG	[82]
C. aurantifolia Swing.	*GUS*	*A. tumefaciens*	[83]
C. paradisi Macf.	*GUS, uncp, gna*	*A. tumefaciens*	[84]
Carrizo citrange	*uid*A and *npt*II	*A. tumefaciens*	[85]
C. sinensis L. Osb.	*GUS*	*A. tumefaciens*	[86]
C. aurantifolia (Christm.) Swing.	*GUS* and *npt*II	*A. rhizogenes*	[2]
C. aurantium L.	*cp*	*A. tumefaciens*	[10]
Tangelo	*GUS* and *npt*II	Particle bombardment	[87]
Carrizo citrange	*GUS* and *npt*II	*A. tumefaciens*	[88]
C. sinensis L. Osb.	*GUS* and *npt*II	*A. tumefaciens*	[89]
C. reticulata Blanco	*GUS*	Electroporation	[90]
Citrus spp.	*GUS* and *npt*II	*A. tumefaciens*	[91]
Citrus spp.	*cat* and *npt*II	PEG	[92]

orange (*C. sinensis* (L.) Osbeck). Plasmid DNA encoding the nondestructive selectable marker enhanced green fluorescent protein gene was introduced using polyethylene glycol into protoplasts of "Itaborai" sweet orange isolated from an embryogenic nucellar-derived suspension culture. Following protoplast culture in liquid medium and transfer to solid medium, transformed calluses were identified via expression of the green fluorescent protein, physically separated from nontransformed tissue and cultured on somatic embryogenesis induction medium. Transgenic plantlets were recovered from germinating somatic embryos and by *in vitro* rooting of shoots [82].

As well as the transformation studies conducted for gene expression, several studies conducted for gene silencing. RNA interference (RNAi) are a posttranscriptional gene-silencing phenomenon induced by double-stranded RNA. It has been widely used as a knockdown technology to analyze gene function in various organisms. Although RNAi was first discovered in worms, related phenomena such as posttranscriptional gene silencing and coat protein-mediated protection from viral infection had been observed in plants prior to this. In plants, RNAi is often achieved through transgenes that produce hairpin RNA. For genetic improvement of crop plants, RNAi has advantages over antisense-mediated

gene silencing and cosuppression, in terms of its efficiency and stability [102]. Soler et al. [32] stated Citrus tristeza virus (CTV), the causal agent of the most devastating viral disease of citrus, has evolved three silencing suppressor proteins acting at intra- (p23 and p20) and/or intercellular level (p20 and p25) to overcome host antiviral defence. Mexican lime was transformed with an intron-hairpin vector carrying full-length, untranslatable versions of the genes p25, p20, and p23 from CTV strain T36 to silence the expression of these critical genes in CTV-infected cells. Three transgenic lines presented complete resistance to viral infection, with all their propagations remaining symptomless and virus-free after graft inoculation with CTV-T36, either in the nontransgenic rootstock or in the transgenic scion. Accumulation of transgene-derived siRNAs was necessary but not sufficient for CTV resistance. Inoculation with a divergent CTV strain led to partially breaking the resistance, thus showing the role of sequence identity in the underlying mechanism. Results are a step forward to developing transgenic resistance to CTV and also show that targeting simultaneously by RNA interference (RNAi) the three viral silencing suppressors appear critical for this purpose, although the involvement of concurrent RNAi mechanisms cannot be excluded.

3. Conclusion

Genetic transformation is an attractive alternative technique for citrus genetic improvement. However, transformation efficiencies are generally low, and protocols are dependent on species, or even cultivar dependent. One of the limitations within this technology is low plant regeneration frequencies especially for many of the economically important citrus species [65]. In addition, difficulty in rooting transgenic shoots for some citrus cultivars has been reported [10, 89, 91]. Development of effective genetic transformants therefore requires specific studies on *in vitro* regeneration conditions for each genotype.

The development of direct genetic manipulation techniques has provided new opportunities for plant improvement. Plant transformation has made it possible to modify just one or two traits, while retaining the unique characteristics of the original cultivar. The characters that could potentially be manipulated by genetic transformation of *Citrus* include pest and disease resistance, growth habit, and fruit quality. In order to use this technology, it is essential to develop efficient genetic transformation systems for *Citrus*. [2].

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Research Article

Micropropagation of *Origanum acutidens* (HAND.-MAZZ.) IETSWAART Using Stem Node Explants

Mehmet Ugur Yildirim

Department of Field Crops, Faculty of Agriculture, Ankara University, Diskapi, 06110 Ankara, Turkey

Correspondence should be addressed to Mehmet Ugur Yildirim; muyildirim72@gmail.com

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Origanum acutidens (HAND.-MAZZ.) IETSWAART is a promising ornamental plant that can be widely used in landscape management. It is endemic to Eastern Anatolian region of Turkey. Tissue culture has not been used to micropropagate it. The study reports stem node explants from one-week-old seedlings of the plant for successful micropropagation. The stem nodes were cultured on MS medium containing 0.6, 1.2, 1.8, and 2.4 mg/L BAP with 0.2 mg/L NAA. Visible effects of culture media on shoot proliferation were recorded. Shoot regeneration rate was maximum on MS medium containing 1.80 mg/L BAP-0.2 mg/L NAA. The micropropagated shoots were rooted on MS medium containing 0.2 mg/L NAA. All microrooted plantlets survived during acclimatisation on peat moss. It was concluded that *O. acutidens* can be successfully micropropagated under *in vitro* conditions.

1. Introduction

Origanum acutidens (HAND.-MAZZ.) IETSWAART is a perennial herbaceous plant endemic to Eastern Anatolian region of Turkey and has beautiful small light pink to white flowers that open during July-August period each year [1]. They grow on limestone and calcareous rocks and slopes need very little water for their growth and maintenance. Sweet scent from leaves is admired since long times and the intensely aromatic leaves are prized to make their great potential for use in urban landscaping and as ornamental border plant in rock gardens. It is also used as aromatic plants, since ancient times for their preservative and medicinal attributes, as well as to impart flavor to foods [2].

There is need to develop methodologies for multiplication and spreading of this plant for the benefit of people. Overexploitation of the plant from natural resources is acting negatively on the populations of the plant and large reserves of the plant that were visible a few years back are no longer visible these days [3]. This suggests that the plant could be used as ornamental in landscaping, pharmaceutical, or food industry after development of protocols for its multiplication through traditional or modern biotechnological methods. Plant tissue culture can act as a possible alternative, which may allow rapid propagation for commercial purpose. In line with this, the study aimed to develop an efficient mass proliferation protocol *O. acutidens* using stem node explants, which has never been reported earlier.

2. Materials and Methods

2.1. Plant Material and Surface Sterilization. The seeds of *O. acutidens* were collected from the botanical gardens of the Department of Field Crops, Ankara University, Ankara, Turkey during 2012. Soon after collection, the seeds were surface sterilised in 5% NaOCl for sterilization. This was followed by 3 × 5 min rinsing with sterilised bidistilled water.

2.2. Isolation of Explants and Determination of the Best Dose for Sterilisation. The seeds were germinated on 35 mL of MS [4] medium supplemented with 30 g/L sugar and solidified with 6.2 g/L agar (Duchefa) for one week. Once the seeds germinated, they were allowed to grow for one week to obtain miniseedlings. The first stem node from the bottom was used as explant. These stem nodes were cultured on 35 mL MS shoot regeneration medium containing 0.6, 1.2, 1.8, and 2.4 mg/L BAP with 0.2 mg/L NAA supplemented with 30 g/L sucrose. Each culture medium was solidified using

Table 1: Effects of various concentrations of BAP-NAA on shoot regeneration from stem node explant of *O. acutiden*.

Regeneration medium		Percentage (%) of shoot regeneration	Number of shoots per explant	Shoot length (cm)
BAP (mg/L)	NAA (mg/L)			
0.6	0.2	86.67[ab]	2.59[d]	0.56[c]
1.2	0.2	86.67[ab]	6.00[c]	0.78[c]
1.8	0.2	100.00[a]	8.45[b]	2.36[a]
2.4	0.2	100.00[a]	9.31[a]	1.81[b]
Control		0.00	0.00	0.00

Values within column followed by different small letters are significantly different at the 0.01 level by Duncan's test. Each value is the mean of 3 replications.

Table 2: Effects of various concentrations of regeneration medium on rooting on MS medium containing 0.2 mg/L NAA.

Shoot Regeneration medium		Percentage (%) of rooting	Mean number of roots per explant	Mean shoot length (cm)
BAP (mg/L)	NAA (mg/L)			
0.6	0.2	100.00	1.00[d]	0.67[d]
1.2	0.2	100.00	1.49[c]	2.22[c]
1.8	0.2	100.00	2.50[a]	5.91[a]
2.4	0.2	100.00	2.00[b]	3.22[b]

Values within column followed by different small letters are significantly different at the 0.05 level by Duncan's test. Each value is the mean of 3 replications.

0.62% (w/v) agar (Duchefa) cultured at 24°C in sterile Magenta GA^7 Vessels under Philips-day light lamps TLD 36 W/54, Hungary with light intensity of 35 μmol m^{-2} s^{-1} under 16 h light photoperiod.

The micropropagated shoots were rooted on MS medium containing 0.2 mg/L NAA supplemented with 30 g/L sucrose and solidified with 6.2 g/L agar for 28 days in Magenta GA^7 Vessels under 16 h light photoperiod.

The pH of shoot and root induction medium was adjusted to 5.7 ± 0.1 with 1 N NaOH or 1 N HCl before autoclaving. All cultures were autoclaved at 121°C, 117.679 kPa for 20 min.

Once the plants rooted, the agar sticking to the roots was removed under tap water and the plantlets were moved to plastic pots containing peat moss. These plants were acclimatised at 20–22°C in the greenhouse under 16 h light photoperiod and 80% humidity.

2.3. Statistical Analysis. The regenerating shoots on each culture medium counted after 45 days to evaluate the regeneration potential of the stem node explants are used in the study. Each experimental treatment had 3 replicates containing 5 explants. Any experimental data taken in percentages were arcsine transformed [5] before statistical analysis. The data related to shoot regeneration was analyzed using one-way ANOVA followed by comparing means using Duncans multiple range test that was performed using statistical software IBM SPSS 20.0 for windows.

3. Results

3.1. Effects of Different Concentrations of BAP-NAA on Shoot Regeneration. The mean percentage of shoot regeneration, number of shoots per explant, and mean shoot length varied in each regeneration medium and showed sharp statistical differences ($P < 0.01$) among them on each concentration of BAP-0.2 mg/L NAA (Table 1). The results showed that different concentrations of BAP-0.2 NAA mg/L are effective combinations for the regeneration of shoots from stem node explants.

The shoot induction started with swelling of explants followed by initiation of shoot buds after one week of culture. These shoot buds gradually developed into fully developed shoots after 45 days of culture. No shoot regeneration was recorded on control (MS medium). Analysis of results showed that shoot regeneration was recorded on all combinations of BAP-NAA with 86.67 to 100% shoot regeneration. Maximum shoot regeneration percentage was recorded on MS medium containing 1.8 mg/L BAP-0.2 mg/L NAA and 2.4 mg/L BAP-0.2 mg/L NAA.

Number of shoots per explant ranged from 2.59 to 9.31 that increased consistently with each increase in the concentration of BAP-0.2 mg/L NAA in MS medium. Each increase in the concentration of BAP-0.2 mg/L NAA had sharp increase in the number of shoots per explant. Minimum and maximum number of 2.59 and 9.31 shoots per explant was recorded on MS medium containing 0.6 mg/L BAP-0.2 mg/L NAA and 2.4 mg/L BAP-0.2 mg/L NAA, respectively.

Shoot length ranged from 0.56 to 2.36 cm that increased consistently from 0.6 mg/L BAP-0.2 mg/L NAA to 1.8 mg/L BAP-0.2 mg/L NAA. Thereafter, with an increase in concentration to 2.4 mg/L BAP-0.2 mg/L NAA, a sharp decline in the shoot length was observed. Minimum and maximum mean shoot length of 0.56 cm and 2.36 cm per explant was recorded on MS medium containing 0.6 mg/L BAP-0.2 mg/L NAA and 1.8 mg/L BAP-0.2 mg/L NAA, respectively.

4. Rooting

Well-developed shoots with mean length of 0.56–2.36 cm were rooted on MS medium containing 0.2 mg/L NAA (Table 2). The results showed that initial length of shoots did not affect rooting. Rooting was noted on all explants. However, initial length of shoots affected root length per explant. The number of roots per explant ranged from 1.00 to 2.50 and

root length had range of 0.67 to 5.91 cm; such that the maximum number of 2.50 roots per shoot with 5.91 cm long roots was noted on shoots regenerated on MS medium containing 1.8 mg/L BAP-0.2 mg/L NAA. It was followed by 2 roots per shoot with 3.22 cm length noted on shoot regenerated on MS medium containing 2.4 mg/L BAP-0.2 mg/L NAA.

These plantlets were transferred to compost contained in culture vessels and incubated in glass house under ambient conditions of temperature and humidity for acclimatisation. All plants transferred to glass house acclimatised with features of growth and flowered.

5. Discussion

There are few reports on mass proliferation of *Origanum*. First study on callus culture of *O. spyleum* was made by Akçam and Yürekli [6]. Tissue culture on *O. bastetanum* [7, 8] and *O. vulgare* [9, 10] has also been carried out previously. However, there is no report on multiplication of the plant through *in vitro* techniques; therefore, propagation technique is of particular importance in *O. acutidens*.

The present study reports the effect of stem node explants on regeneration for developing an easy and reliable protocol for shoot regeneration explants.

It is thought that the variants of BAP-NAA may have variable effect on the regeneration and rooting. In line with the hypothesis, the experimental results showed that any concentration of BAP-NAA was suitable for regeneration. It is thought that explants cultured on MS medium contain 0.6–1.8 mg/L BAP-0.2 mg/L NAA. The higher concentration 2.4 mg/L BAP-0.2 mg/L NAA induced negative competition for nutrients due to regeneration of more shoots resulting in reduced length of shoots, whereas the lower concentrations 0.6–1.2 mg/L BAP-0.2 mg/L NAA induced inhibition due to low number and reduced length of shoots.

The shoots regenerated on BAP-NAA were easily rooted on MS medium in agreement with Socorro et al. [7] and Goleniowski et al. [9]. No abnormality was recorded in the rooted and acclimatized plantlets. This confirms that *in vitro* regenerated *O. acutidens* plantlets could be effectively used for regeneration. Goleniowski et al. [9] reported spontaneous rooting in shoot multiplication medium supplemented with BA (0.28 μM) + NAA (0.53 μM) for *O. vulgare,* whereas Socorro et al. [7] reported on rooting of micropropagated plantlets of *O. bastetanum,* on peat substrate. During the present investigation we obtained rooting in 96% of shoots (an average root length of 5.52 ± 0.2) on medium containing in 0.5 mg/L IBA.

In conclusion, the results showed that *in vitro* production *O. acutidens* is possible and this plant could be successfully utilized for *in vitro* commercial propagation. It is evident that *in vitro* shoot regeneration and rooting in *O. acutidens* are no longer a problem.

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Research Article

The Influence of 1-Triacontanol on the Growth, Flowering, and Quality of Potted *Bougainvillea* Plants (*Bougainvillea glabra* var. "Elizabeth Angus") under Natural Conditions

Mohammad Moneruzzaman Khandaker, Golam Faruq, M. Motior Rahman, M. Sofian-Azirun, and Amru Nasrulhaq Boyce

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Correspondence should be addressed to Mohammad Moneruzzaman Khandaker; kmoneruzzaman@yahoo.com

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Selected physiological and biochemical parameters were monitored at the vegetative and reproductive growth stages in potted *Bougainvillea* plants treated with five different concentrations of TRIA. Advanced flowering, flower bud number, and blooming rate increased significantly with 0.5 and 1.0 mg/L TRIA treatments. Similarly, photosynthetic rate, pigment content, quantum yield, and stomatal conductance increased significantly with 2.5, 1.0, and 5.0 mg/L TRIA treatments. Higher levels of N, P, and K, as well as increased total soluble solids (TSS) and higher sugar and protein contents, were recorded in treated plants. Furthermore, 46% more flowers, a 1.5-fold increase in bract weight, increased longevity, and 40% less leaf abscission were recorded following 2.5 mg/L TRIA treatment. Phenol and flavonoid contents, sucrose phosphate synthase (SPS), and antioxidant activities were also markedly increased with 2.5 and 1.0 mg/L TRIA treatments. However, ethylene production was significantly lower in the treated plants. Positive correlations were observed between leaf TSS and flowering time and flower number, between leaf sugar content and bract weight, and between net photosynthesis and bract growth and dry matter production. It can be concluded that the foliar spray of TRIA stimulates growth, enhances flowering, and improves the quality of potted *Bougainvillea* plants.

1. Introduction

Bougainvillea is a flowering ornamental plant that belongs to the family Nyctaginaceae (i.e., Four-O'Clock), which has 18 species and is an economically important ornamental flower in tropical and subtropical regions. The true, perfect flower is small, tubular, commonly white or yellow, and surrounded by showy, vibrantly colored petaloid bracts. The decorative quality of potted flowering plants depends on their flower and leaf number, bract color, longevity, and turnover. The commercial value of *Bougainvillea*, as for many potted plants, is seriously affected by the abscission of leaves and flowers, and this value can be improved by prolonging bract longevity and increasing its quality. In our previous study, it has been reported that *Bougainvillea* bracts are abscised within 4 to 5 weeks after blooming [1]. Darnell et al. [2] reported that flowering in many species can be induced by a variety of environmental techniques and the application of growth-promoting chemicals.

Growth regulators play integral roles in controlling the growth, development, metabolism, and morphogenesis of flowering plants [3]. Gibberellic acid (GA_3) was demonstrated to induce inflorescence development and flowering and to increase the number of flowers [4]. The application of kinetin and the removal of young leaves enhanced inflorescence development and improved the quality of *Bougainvillea* bracts [5]. In the present study, we tested the effects of TRIA, as well as other growth regulators, on the physiological activities and flowering behavior of *Bougainvillea* plants. TRIA, a saturated primary alcohol (n-C30H61OH), is a natural component of plant epicuticular waxes that can enhance plant growth [6, 7]. Many researchers have reported the positive role of TRIA in enhancing growth, yield, photosynthesis, nitrogen fixation, enzymatic activities,

and levels of free amino acids, reducing sugars, and soluble protein [8]. TRIA application increased plant dry weight, protein and chlorophyll contents, and the net photosynthetic rate in rice [9]. It has also been reported that TRIA is involved in the upregulation of many genes that are involved in photosynthesis. Skogen et al. [10] reported that TRIA application increases plant growth, the number of inflorescences, and the quality of chrysanthemum (*Chrysanthemum morifolium*) flowers. Many researchers have examined the effects of TRIA on vegetables and certain agronomic crops, and these studies reported stimulatory effects on crop growth, yield, and quality. It is believed that the growth regulator TRIA could also have stimulatory effects on flowering plants. However, very few studies have examined how to improve the quality and longevity of *Bougainvillea* plants under natural or green house conditions.

Currently, no information is available in the literature on the effects of TRIA on plant growth, flowering, and quality in potted *Bougainvillea* plants. This study evaluated the impacts of TRIA on improving the quality of *Bougainvillea* plants under natural conditions. It is proposed that TRIA application can improve the plant's physiological activities, stimulate flowering, and increase the quality of potted *Bougainvillea* plants. The findings of this study will provide a basis for future research into the growth-regulating effects of TRIA on *Bougainvillea* and other ornamental and flowering plants.

2. Materials and Methods

2.1. Experimental Site and Plant Material. The experiments were carried out at the Plant Physiology Garden at the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, between 2010 and 2011. The experiments during the first season (June 2010–October 2010) and the second season (February 2011–June 2011) were performed in the same location. One-year-old seedlings of potted *Bougainvillea* plants were collected from a commercial nursery in Sungai Buloh, Selangor when the plants were 0.6 m long with approximately 6 to 8 secondary branches. These seedlings were planted in 7-inch pots filled with garden soil and peat soil in a ratio of 5 : 5. The plants were thoroughly watered when the soil appeared dry, approximately every 3 to 5 days, during the experimental period. All of the experiments, regardless of the season or year, were performed under the following normal prevailing conditions for this region: temperature 21–32°C, maximum PAR 2000 μE m^{-2} s^{-1}, and relative humidity of 60%–90%. Five grams of nutrients (N : P: K, at a ratio of 12 : 12 : 17) per plant were applied at 15-day intervals. Twenty *Bougainvillea* plants were used for each season. A completely randomized design (CRD) with four replications was used for each season's experiment.

2.2. Treatment Setting. A total of sixty uniform branches (three per plant) were selected for the treatment application in each season, and a single plant was taken as an experimental unit. Every branch was tagged 15 cm below the apex of the branch. The experiments consisted of five treatments (0, 0.5, 1.0, 2.5, and 5.0 mg/L TRIA), including the control, with four replications and three subreplications (12 replicates for each treatment). The treatments were applied at both the vegetative shoot and the reproductive stages, which exhibited strong growth with prolific and dense leaves. The selected branches were sprayed twice per week until inflorescence development. A total of ten spray applications were performed, five times before flowering and five times after flowering; 60 mL TRIA solution was used per treatment (twelve branches).

2.3. Growth Rate, Bract Length, Bract Weight, Blooming Rate, and Longevity of Bougainvillea. The leaf and shoot growth rates, flower bud number, blooming rate, bract length, and shoot elongation were measured at three-day intervals. Individual bract and flower weights, as well as bract, flower longevity, and leaf drops, were measured after 15 days of observation. All of the growth rates were measured using a vernier scale, and the growth per day (in cm) was calculated. Close observations were made to determine the number of nodes before the first inflorescence for each treatment. Individual bract and bract cluster weight, including the flowers, fresh biomass, and dry biomass, were measured using a Mettler PJ3000 balance, and bract lengths were measured on a Mitutoyo Vernier Scale. The dry matter content was measured in 0% moisture conditions. From the beginning of the experiments, 12 buds per treatment were selected for full blooming and longevity measurements. Three branches per treatment were selected for leaf abscission measurements. The observations were made when all of the bracts were open and abscission had occurred.

2.4. Mineral Content, Photosynthetic Pigment Levels, Quantum Yield, Photosynthetic Rate and Stomatal Conductance Measurements. The nutrient content of *Bougainvillea* leaves (N and P) was analyzed using a multielement analyzer (MEA). Grounded leaf samples were mixed with water, and 1 mL of the sample extract was injected into the MEA for the calculation. The potassium (K^+) content of the leaves was determined using a Cardy potassium meter. The chlorophyll and carotene contents of the *Bougainvillea* leaves were determined using the methods described by Hendry and Price [11]. Chlorophyll fluorescence yield was measured using a Plant Efficiency Analyzer (Hansatech Instruments Ltd., England). Optimum quantum yield is presented as Fv/Fm, where Fv = relative variable fluorescence (Fm-Fo) and Fm = maximum fluorescence. The instrument was run at 27°C, with a time range of 10 μs to 3 sec. The photosynthetic rate (Pn) of the *Bougainvillea* plants was measured using a portable photosynthesis system (Li-6400XT, Li-COR, USA), and the measurements were performed according to the methods described by Khandaker et al. [12]. Stomatal conductance was measured using a portable Porometer (Leaf Porometer, Model SC-1, USA). A leaf chamber was attached to one leaf and kept the leaf at an ambient temperature for 10–15 min to maintain sunlight adaptation. Stomatal conductance was measured in three replicates from different spots on a single leaf.

2.5. TSS, Total Sugar Levels, Protein Content, Phytochemical Constituents, SPS Activitys and Ethylene Production. The total soluble solids (TSS) content of the leaves was evaluated

at 25°C with an Atago 8469 hand refractometer (Atago Co. LTD., Tokyo, Japan) and was expressed as °Bx. The total soluble sugar level of the leaves was determined using the phenol-sulphuric method [13]. Leaf protein content of the treated and nontreated branches was determined according to the Bradford method [14]. Antioxidant capacity was determined via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Tadolini et al. [15]. The total phenolic contents of the *Bougainvillea* leaves were determined using the Folin-Ciocalteu assay, as described by Singleton and Rossi [16]. The total flavonoid content was determined using the aluminum chloride colorimetric assay, using catechin as a standard [17]. Sucrose phosphate synthase activity was assayed under the Vmax condition according to the methods of Huber et al. [18]. The ethylene production of the *Bougainvillea* plants was measured during the vegetative and reproductive growth stages. Four leaves that were previously treated with TRIA were detached from four sample plants for each treatment stage, one at the vegetative growth stage and one more each on the 1st, 5th, and 10th days after flowering. The leaves were then incubated in a 25 mL sealed plastic jar for 2 h at temperatures ranging from 20°C to 28°C. Following the incubation, air samples were taken from the headspace for ethylene analysis using a Shimadzu GC-14A gas chromatograph. The ethylene production rates were calculated and expressed as nL g^{-1} h^{-1}.

2.6. Statistical Analysis. A completely randomized design (CRD) with four replications (3 subreplications) was used for the treatments in both seasons' experiments. The data from both seasons were pooled and analyzed using MSTAT-C statistical software. The one-way ANOVA was applied to evaluate significant differences in the parameter studied for the different treatments. The least significant difference (LSD) and honestly significant difference (HSD) were calculated following a significant F-test (at $P = 0.05$).

3. Results

Table 1 shows the time course growth of *Bougainvillea* plants under natural conditions. TRIA treatments significantly affected the vegetative growth of *Bougainvillea* plants (Table 1). A faster growth rate was observed in leaf area and shoot length in all treatments when compared to the control group. The growth rate, as measured by shoot length, was 44% higher in the 2.5 mg/L TRIA-treated plant than in the control. This result was statistically significant, although no significant difference was observed for this parameter between the 2.5 mg/L group and the other TRIA treatment groups (Table 1). Shoot diameter growth was not significantly affected by TRIA treatments. The results indicated that flower bud and bract growth length were also significantly affected by TRIA treatments. Flower bud growth was higher in the 1.0, and 2.5 mg/L TRIA treatment groups than in the other treatment groups and the control group. This growth trend was observed during the entire reproductive period and resulted in a larger flower bud. The bract length growth rate was nearly 30% to 50% higher in all of the treatments when compared to the control group, and these differences between the treatments and the control were statistically significant (Table 1). Bract diameter growth was not significantly affected by TRIA treatments.

As shown by the results presented in Figure 1, TRIA treatments significantly increased the number of flower buds per 15 cm of branch. On the 12th day of observations, the flower bud number was 55%, 44%, and 35% higher in the 0.5, 1.0, and 2.5 mg/L TRIA treatment groups, respectively, compared to the control group. The most significant differences were observed when the branches were treated with 0.5 and 1.0 mg/L TRIA. This increasing trend in bud number was recorded throughout the entire observation period during the two seasons' experiments.

The results demonstrated that TRIA treatments significantly affected the bract blooming of *Bougainvillea* plants (Figure 2). It was observed that bract blooming increased for up to 2 weeks following bud development and thereafter decreased in the treated and untreated branches (Figure 2). By the 9th day of observation, the 0.5 mg/L TRIA treatment had produced the highest number of bract blooms (8) followed by the 1.0, 2.5, and 5.0 mg/L TRIA treatments, which resulted in 7, 7, and 5 bract blooms, respectively. The control plants produced the lowest number (3) of blooming bracts.

The application of TRIA markedly increased the vegetative growth of *Bougainvillea* plants (Figure 3). The shoot growth rate in all of the treated plants was significantly higher throughout the experimental periods and was 32% higher in 5.0 mg/L TRIA-treated plants compared to the untreated group on the 3rd day of observations. It was also noted that the shoot growth rate increased with TRIA concentrations, and a relatively slow growth rate was observed in the control plants.

The accumulation of mineral content in the leaves of *Bougainvillea* was significantly affected by treatment with TRIA (Table 2). The results demonstrated that the 2.5 mg/L TRIA treatment resulted in the accumulation of 39%, 37%, and 35% more N, P, and K, respectively, when compared to control plants. The different treatments varied significantly among themselves only with respect to N accumulation in the leaves. TRIA treatment significantly affected the stomatal conductance of *Bougainvillea* leaves. The highest stomatal conductivity (38 m^2s/mol) was observed in the 2.5 mg/L TRIA-treated leaves, followed by the other treatments and the control. The lowest stomatal conductivity (25 m^2s/mol) was recorded in the control sample (Table 2). *Bougainvillea* plants that were treated with TRIA exhibited increased fresh and dry biomass production (Table 2). We found that the branches that were treated with 2.5 or 1.0 mg/L TRIA appeared to be healthier than those of the control plants and produced 1.28- or 1.23-fold more dry matter than the control plants, respectively. The next strongest effects were observed for the 5.0 and 0.5 mg/L TRIA treatments, and the branches from the control plants exhibited the lowest dry weights.

Leaf chlorophyll content, which indirectly indicates the health status of a plant, was significantly affected by TRIA treatments in both seasons (Table 3). In the first season, the highest amounts of leaf chlorophyll a, b, and $a + b$ content (2.7, 1.40, and 4.10 mg g^{-1} FW) were recorded for the 2.5 mg/L TRIA-treated plants followed by the other treatments and the

Table 1: The effects of TRIA treatments on the growth rates of leaves, shoots, flower buds, and bracts of *Bougainvillea glabra* under natural conditions.

Treatment (mg/L)	Growth rate (cm/day): length and diameter							
	Leaf		Shoot		Flower bud		Bract	
	Length	Diam.	Length	Diam.	Length	Diam.	Length	Diam.
Control	0.12^{b}	0.03^{b}	0.16^{b}	0.01^{a}	0.19^{d}	0.05^{b}	0.12^{c}	0.08^{a}
TRIA 0.5	0.18^{a}	0.04^{b}	0.20^{a}	0.01^{a}	0.28^{b}	0.07^{a}	0.15^{b}	0.10^{a}
TRIA 1.0	0.20^{a}	0.06^{a}	0.21^{a}	0.02^{a}	0.38^{a}	0.10^{a}	0.18^{a}	0.11^{a}
TRIA 2.5	0.17^{a}	0.08^{a}	0.23^{a}	0.02^{a}	0.33^{a}	0.12^{a}	0.19^{a}	0.11^{a}
TRIA 5.0	0.19^{a}	0.07^{a}	0.21^{a}	0.02^{a}	0.25^{c}	0.10^{a}	0.17^{b}	0.10^{a}
$LSD_{0.05}$	**0.06**	**0.05**	**0.05**	**ns**	**0.06**	**0.05**	**0.06**	**ns**

Values in a column sharing the same lower case letters are not significantly different at $P < 0.05$ (LSD test).

[a, b, c, d]Means within the same column followed by the same letter do not differ significantly according to the LSD test at $P < 0.05$; ns, not significant.

Table 2: The effects of TRIA treatments on the mineral content, stomatal conductance, and the fresh and dry biomasses of *Bougainvillea glabra* under natural conditions.

Treatment (mg/L)	Mineral ion content (mg/g)			Stomatal cond.	Fresh Wt of	Dry Wt of branch (g)
	N	P	k	(m^2s/mol)	branch (g)	(dry matter)
Control	2.18^{c}	0.29^{b}	0.95^{b}	25^{c}	31.85^{c}	19.85^{d}
TRIA 0.5	2.80^{b}	0.35^{a}	1.20^{a}	33^{b}	34.30^{b}	21.30^{b}
TRIA 1.0	2.68^{b}	0.38^{a}	1.23^{a}	34^{a}	37.50^{a}	24.50^{a}
TRIA 2.5	3.06^{a}	0.40^{a}	1.29^{a}	38^{a}	39.30^{a}	25.30^{a}
TRIA 5.0	2.78^{b}	0.35^{a}	1.28^{a}	37^{a}	35.30^{a}	22.50^{a}
$LSD_{0.05}$	**0.24**	**0.17**	**0.19**	**4.6**	**3.9**	**2.64**

Values in a column sharing the same lower case letters are not significantly different at $P < 0.05$ (LSD test).

[a, b, c, d]Means within the same column followed by the same letter do not differ significantly according to the LSD test at $P < 0.05$.

untreated control group. In the second season, the 2.5 mg/L TRIA treatment also produced 25%, 22%, and 23% more chlorophyll *a*, *b*, and *a* + *b* contents, respectively, compared to the control plants. The 2.5 mg/L treatment was followed by the 1.0, 5, and 0.5 treatments, and the control plants exhibited the lowest chlorophyll contents. The carotenoid content of the leaves was also significantly affected by TRIA treatments (Table 3). The leaf carotenoid content was 26% and 30% higher in the 2.5 mg/L treated branches, compared to the control plants for the 1st and 2nd year observations, respectively. In all the treated plants, the leaf carotenoid content was higher in the treated than in the untreated plants, and the carotenoids in the leaves of *Bougainvillea* increased with flowering advance (data not shown). The photosystem of the leaves, as measured by quantum yield, had lower values in the nontreated plants; significantly higher quantum yield values were observed during the two years of study in all the TRIA-treated *Bougainvillea* plants compared to the untreated plants (Table 3).

The photosynthetic activity of *Bougainvillea* plants was determined, in terms of μmol CO_2 fixation $m^{-2}s^{-1}$, to measure the activity level of the photosynthetic carbon metabolism. In this study, TRIA treatments were observed to significantly increase the leaf photosynthetic activity in both growing seasons. In the first year, at 350 ppm CO_2 and light intensities of 400, 800, 1200, and 2000 μmolm$^{-2}s^{-1}$, the photosynthetic activities were 1.6-, 1.7-, 1.8-, and 1.6-fold higher, respectively, in the 2.5 mg/L TRIA-treated leaves than in the control leaves (Figure 4(a)). Similarly, in the 2nd year,

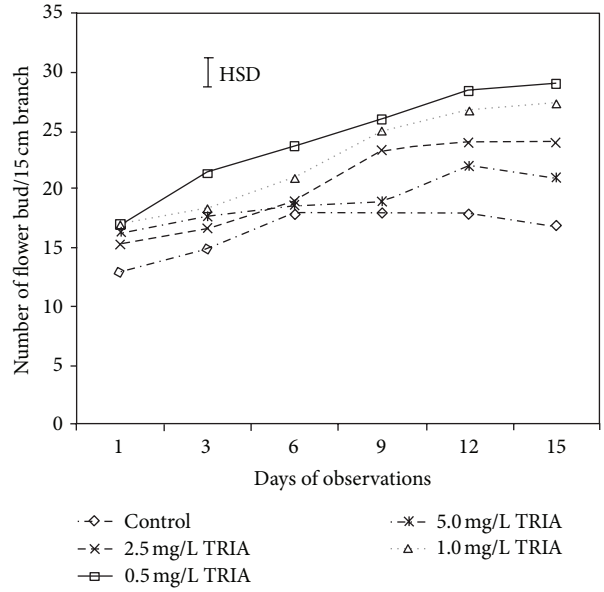

Figure 1: The effect of TRIA treatments on the flower bud formation of *Bougainvillea glabra* under natural conditions.

under the same conditions, the photosynthetic activities were 1.6-, 1.4-, 1.5-, and 1.6-fold higher, respectively, in the 2.5 mg/L TRIA-treated leaves (Figure 4(b)). It was also observed that the 2.5 and 1.0 mg/L TRIA-treated plants exhibited the highest photosynthetic activities in both seasons, while the control

Table 3: The effects of TRIA on the photosynthetic pigments, carotenoids, and quantum yield of *Bougainvillea glabra* under natural conditions.

Treatment (mg/L)	Chl. *a* mg g^{-1} FW	Chl. *b* mg g^{-1} FW	Chl. *a* + *b* mg g^{-1} FW	Carotenoids mg g^{-1} FW	Quantum yield
July 10 to Oct/10					
Control	2.23^{c}	1.18^{d}	3.41^{c}	1.03^{c}	0.80^{b}
TRIA 0.5	2.48^{b}	1.30^{b}	3.78^{b}	1.20^{b}	0.83^{a}
TRIA 1.0	2.65^{a}	1.39^{a}	4.05^{a}	1.35^{a}	0.84^{b}
TRIA 2.5	2.70^{a}	1.40^{a}	4.10^{a}	1.30^{a}	0.87^{a}
TRIA 5.0	2.58^{a}	1.26^{c}	3.84^{a}	1.28^{a}	0.85^{a}
$LSD_{0.05}$	**0.15**	**0.09**	**0.28**	**0.10**	**0.05**
Feb/11 to June/11					
Control	1.95^{d}	1.05^{c}	3.00^{c}	1.08^{c}	0.80^{b}
TRIA 0.5	2.10^{c}	1.25^{a}	3.35^{b}	1.27^{b}	0.82^{a}
TRIA 1.0	2.31^{a}	1.32^{a}	3.63^{a}	1.39^{a}	0.81^{a}
TRIA 2.5	2.43^{a}	1.28^{a}	3.71^{a}	1.40^{a}	0.85^{a}
TRIA 5.0	2.23^{b}	1.20^{b}	3.43^{b}	1.27^{a}	0.83^{a}
$LSD_{0.05}$	**0.13**	**0.08**	**0.14**	**0.08**	**0.06**

Values in a column sharing the same lower case letters are not significantly different at $P < 0.05$ (LSD test).

$^{a, b, c, d}$Means within the same column followed by the same letter do not differ significantly according to the LSD test at $P < 0.05$.

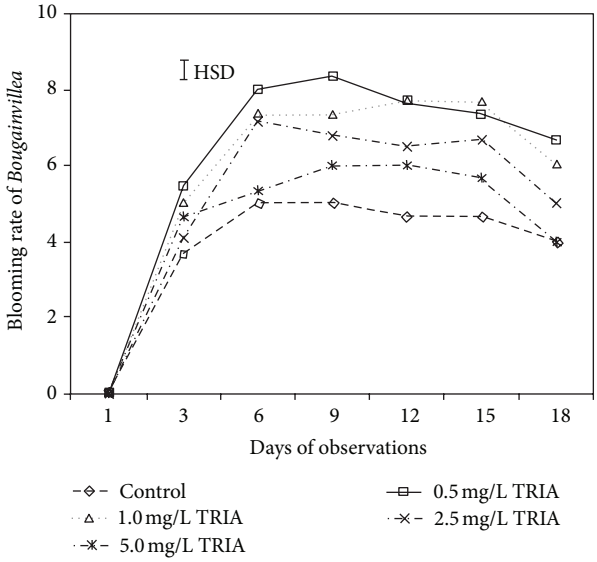

Figure 2: The effect of TRIA treatments on blooming rate of *Bougainvillea glabra* under natural conditions.

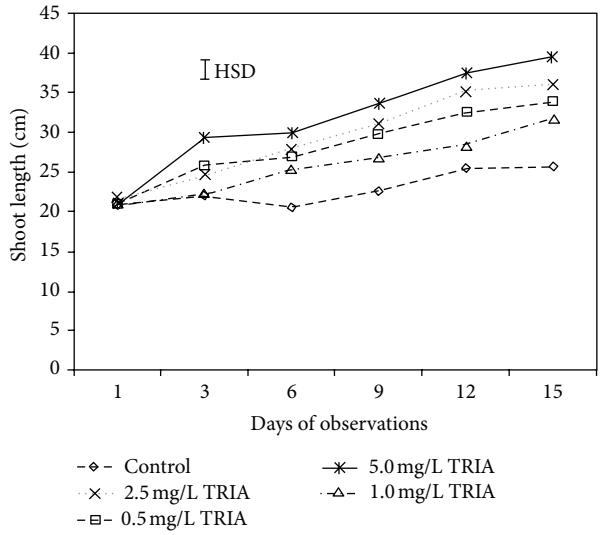

Figure 3: The effect of TRIA treatments on shoot length of *Bougainvillea glabra* under natural conditions.

plants exhibited the least photosynthetic activity (Figures 4(a) and 4(b)).

Treatment with TRIA during the vegetative shoot stage resulted in an advanced flowering of the *Bougainvillea* plants. The results indicated that the 2.5, 5.0, and 1.0 mg/L treated branches produced flowers 12, 11, and 7 days earlier when compared to the untreated control branches, respectively. The differences between the treatment groups and the control group were statistically significant (Table 4). In the present study, it was observed that the TRIA-treated branches produced flowers following the appearance of approximately six fewer nodes compared to the control plants, and this was statistically significant between the treated and the control groups. Treatment with TRIA also increased the flower number significantly when compared to the control plants (Table 4). The highest number of flowers (13) was observed in a 2.5 mg/L treated branch, whereas the control branches produced the lowest number of flowers (7) per branch. As shown in Table 4, the TRIA treatments significantly increased the size and weight of *Bougainvillea* bracts, including the flowers. The highest bract weight (49 g), including the flower, was recorded in the 2.5 mg/L TRIA-treated plant, followed by the other treatments. The control branches exhibited the lowest bract and flower weights (approximately 39 g) (Table 4).

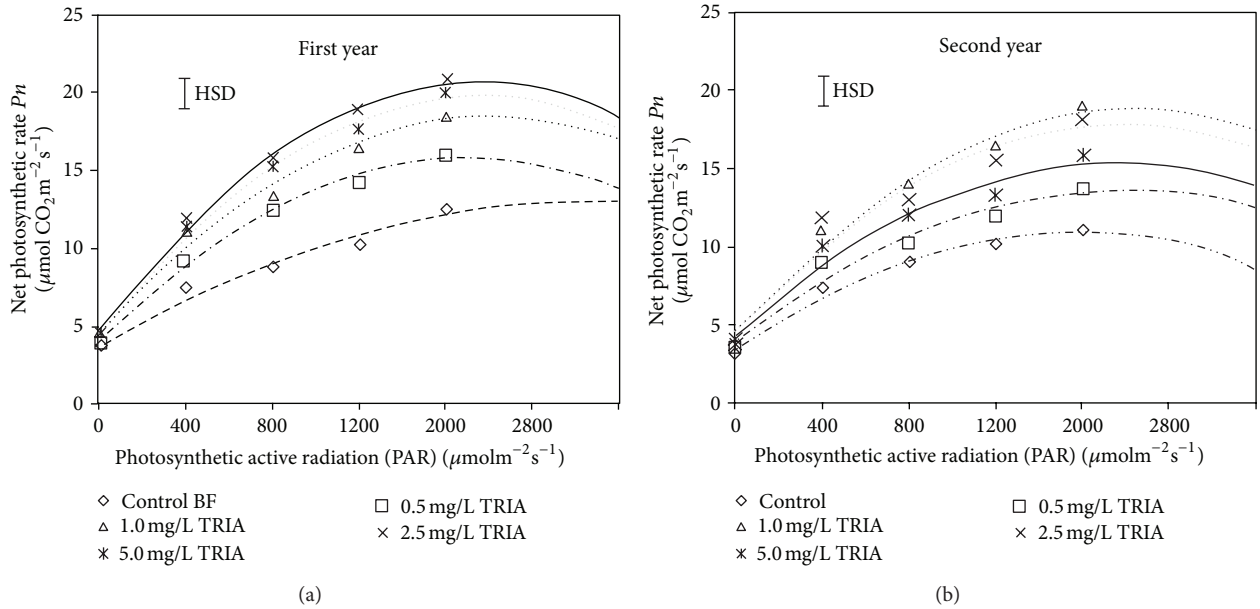

Figure 4: The effect of TRIA treatment on the net photosynthesis rate of *Bougainvillea glabra* under natural conditions.

Table 4: The effect of TRIA treatments on flowering initiation, quality, and the abscission of bracts and leaves of *Bougainvillea glabra* under natural conditions.

Treatment (mg/L)	Flowering required day	Node of first flower bud	Flowers per branch	Fresh weight of flowers	Flower longevity	Leaf drop at 15 DAF
Control	29[a]	20[a]	7[c]	39[b]	29[c]	20[a]
TRIA 0.5	23[b]	14[b]	11[a]	45[a]	33[b]	15[b]
TRIA 1.0	22[b]	16[b]	12[a]	47[a]	35[a]	14[b]
TRIA 2.5	17[d]	14[b]	13[a]	49[a]	38[a]	12[b]
TRIA 5.0	18[c]	13[c]	10[b]	47[a]	35[a]	13[b]
$LSD_{0.05}$	**4.7**	**2.4**	**2.3**	**6.8**	**3.2**	**3.8**

Values in a column sharing the same lower case letters are not significantly different at $P < 0.05$ (LSD test).
[a, b, c, d]Means within the same column followed by the same letter do not differ significantly according to the LSD test at $P < 0.05$.

Foliar TRIA application increased the bract longevity significantly in *Bougainvillea* plants (Table 4). Our results indicated that treatment with 2.5 mg/L TRIA increased the bract longevity by approximately nine (9) days, followed by the 1.0 and 0.5 mg/L TRA treatments, which increased bract longevity by six (6) and four (4) days, respectively, compared to the control group. In this study, it was also observed that TRIA treatments at the vegetative shoot and reproductive stages reduced the leaf drop of *Bougainvillea*. Leaf drop was reduced by 40% in the plants treated with 2.5 mg/L TRIA, compared to the control plants, as observed 15 days after flowering (Table 4).

In this study, it was found that the TSS content of the *Bougainvillea* leaves significantly differed between the treated and the control leaves (Table 5). The highest TSS value (19.3°Bx) was observed in the branches treated with 2.5 mg/L TRIA, followed by the 1.0, 5.0 and 0.5 mg/L treatments. The lowest TSS value (16.5°Bx) was observed in the control branches. On the basis of two years of results, it was observed that TRIA application had a significant effect on the sugar content of *Bougainvillea* leaves. The leaf sugar content was 58% higher in the 2.5 mg/L TRIA-treated plants than in the control plants. The differences between the treatment and control groups were clearly significant ($P < 0.05$), whereas the differences in sugar content between the treatment groups were not statistically significant (Table 5). The same trend was also observed during the 2nd year (Table 5). Various concentrations of TRIA significantly affected the concentrations of phenols and flavonoids and the antioxidant activities of *Bougainvillea* plants (Table 5). Our results indicated that the 1.0 mg/L TRIA treatment yielded 1.15-, 1.14-, and 1.34-fold increases in the phenol and flavonoid contents and the antioxidant activities, respectively, compared to the untreated control group. The next strongest effects were observed for the 2.5, 5.0, and 0.5 mg/L TRIA treatments. The results were statistically significant between the treatment groups themselves and between the treatment and control groups. The soluble protein content of the *Bougainvillea* leaves was significantly affected by TRIA treatments (Table 5). The protein content was 18% higher in the 2.5 mg/L TRIA-treated leaves compared to the control group.

Table 5: The effects of TRIA on phytochemical levels and antioxidant activity in *Bougainvillea glabra* leaves.

Treatment (mg/L)	TSS (°Brix)	Total sugar		Phenol (mg/g)	Flavonoid (mg/g)	Antioxidant (DPPH mg/g)	Soluble protein (mg/g)
		1st Y	2nd Y				
Control	16.5[d]	0.28[b]	0.26[b]	2.94[c]	4.76[d]	223[b]	6.12[c]
TRIA 0.5	17.8[c]	0.33[a]	0.30[a]	3.18[b]	4.93[c]	258[b]	7.15[a]
TRIA 1.0	19.2[a]	0.39[a]	0.33[a]	3.39[a]	5.43[a]	300[a]	6.85[b]
TRIA 2.5	19.3[a]	0.42[a]	0.35[a]	3.26[a]	5.20[b]	312[a]	7.25[a]
TRIA 5.0	18.5[b]	0.40[a]	0.31[a]	3.30[a]	5.26[b]	264[b]	7.17[a]
$LSD_{0.05}$	**1.40**	**0.5**	**0.5**	**0.14**	**0.08**	**41.4**	**0.34**

Values in a column sharing the same lower case letters are not significantly different at $P < 0.05$ (LSD test).

[a, b, c, d]Means within the same column followed by the same letter do not differ significantly according to the LSD test at $P < 0.05$.

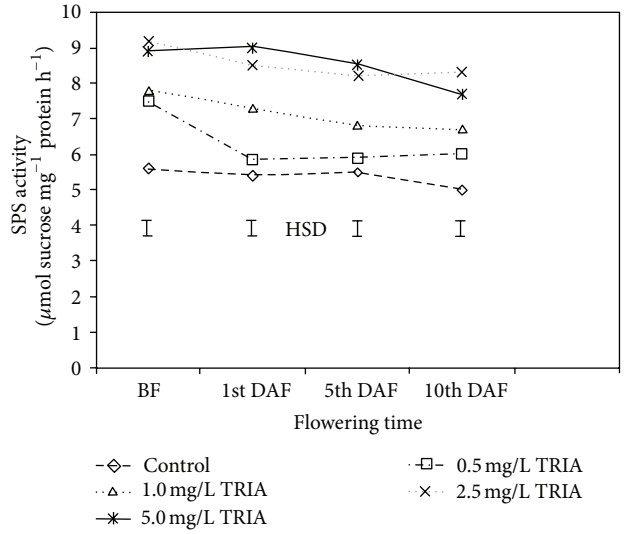

Figure 5: The effect of TRIA treatments on sucrose phosphate synthase (SPS) activity of *Bougainvillea glabra* leaves. BF: before flowering, DAF: day after flowering.

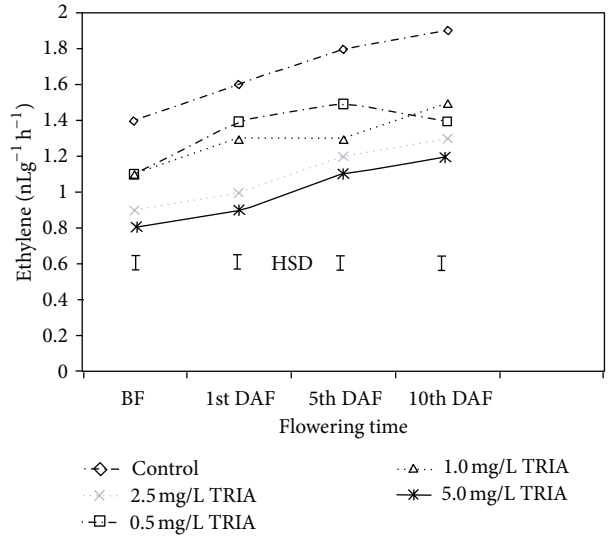

Figure 6: The effect of TRIA treatments on ethylene production of *Bougainvillea glabra* leaves. BF: before flowering, DAF: day after flowering.

In the present study, the activity of sucrose phosphate synthase in the leaves of *Bougainvillea* plants was determined at the vegetative and reproductive growth stages. The results indicated that TRIA treatments significantly elevated the level and activity of the SPS enzyme in both growth stages (Figure 5). At the vegetative shoot stage, the SPS activity increased approximately 1.76-, 1.71-, 1.39- and 1.33-fold in the 2.5, 5, 1, and 0.5 mg/L TRIA treatment groups, respectively, compared to the control group. Accordingly, on the 1st, 5th, and 10th days of flowering, higher sucrose phosphate synthase activities were recorded in the treated plants then in the untreated group (Figure 5). It was also observed that SPS activities began to decrease with flowering time continued and that this trend was stronger in the control plants than in the treated plants.

Treatment with TRIA significantly reduced the ethylene production rate of *Bougainvillea* plants (Figure 6). During the vegetative shoot stage, the untreated leaves contained more ethylene ($1.4\,nLg^{-1}\,h^{-1}$) than the treated plants, with the 2.5, 5, 1 and 0.5 mg/L treated leaves containing 0.8, 0.9, 1.1, and $1.1\,nLg^{-1}\,h^{-1}$ ethylene, respectively. During the 5th day of flowering, the ethylene production rate was 38%, 33%, 27%, and 16% lower in the 5, 2.5, 1, and 0.5 mg/L TRIA treatment groups, respectively, compared to the control group. It was also observed that the ethylene production rate increased with the flowering advance as well as with the age of the *Bougainvillea* plants, and the production rate was significantly lower in the treated plants compared to the control plants (Figure 6).

In this two-year study, it was observed that several parameters were correlated in the TRIA-treated plants. A high degree of correlation was observed between the number of flowers and the leaf TSS ($r = 0.80$), as well as between the flowering required day and the leaf TSS ($r = 0.92$) (Figures 7(a) and 7(b)). Moreover, the flower weight (bract) was correlated with leaf sugar content ($r = 0.93$) as well as bract growth ($r = 0.93$), and dry matter production ($r = 0.89$) was positively correlated with the net photosynthesis rate of the treated plants (Figures 8(a), 8(b), and 8(c)). Furthermore, flower longevity was negatively correlated ($r = 0.86$) with ethylene production in *Bougainvillea* plants (Figure 8(d)).

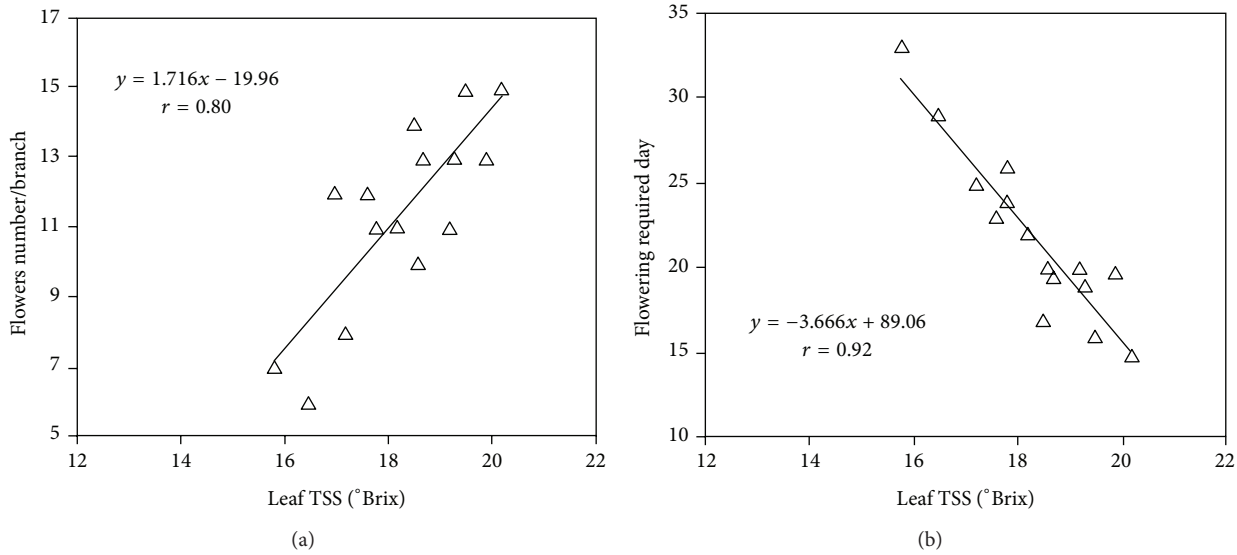

Figure 7: Regression lines for the relationship between flower number and leaf TSS (a) and flowering day and leaf TSS (b) in the treated *Bougainvillea* plants.

4. Discussion

Triacontanol (TRIA) is a saturated long-chain alcohol that is known to have growth-promoting activities on a number of plants when applied exogenously. As an important growth regulator, TRIA has attracted much attention with respect to typifying its physiological effects on a number of agronomic crops. TRIA regulates various growth and developmental processes in plants under both normal and stress conditions [19]. Plants are sensitive to extremely low doses of TRIA; therefore, low concentrations of TRIA may be biologically active [20]. It has been reported that TRIA application enhances water uptake, cell division, cell elongation, and the permeability of plant cell membranes [21]. Our two-year study demonstrated that TRIA treatment increased leaf area, shoot length, flower bud, and bract growth of *Bougainvillea* plants. Reddy et al. [22] also reported that TRIA treatment increased the number of nodes, leaves, and shoots of *Capsicum frutescens* and *Decalepis hamiltonii*. Foliar application of 0.5 mg dm^{-3} TRIA significantly promoted the onset of flowering in green gram plants [23]. In this study, TRIA treatment stimulated bud formation and increased flower budding flower growth and the blooming rate of *Bougainvillea* plants. Skogen et al. [10] reported that TRIA treatment increased the growth, the number of inflorescences, and the quality of *Chrysanthemum morifolium* flowers. These authors also observed that the number of superior quality flowers was nearly doubled in the treated plants compared to control plants. TRIA may provide an active ingredient for bud formation, bud development, and the improved quality of flowers. Moreover, TRIA has been shown to increase the growth and/or yield of the majority of the major annual vegetables and agronomic crops as well as of forest species [24].

The results related to growth and biomass production of *Bougainvillea* plants are consistent with the findings of Muthuchelian et al. [25], who reported that TRIA treatments increased the root and shoot length, leaf density and area, and fresh and dry biomass accumulation of *Erythrina variegata* plants. These effects may be due to the rapid translocation of TRIA throughout the plant, causing a cascade of metabolic events and resulting in significant increases in growth and dry matter [26]. Naeem et al. [8] reported that foliar TRIA application significantly increased the N, P, K, and Ca contents of the leaves of hyacinth bean plants. The findings of the present study also demonstrate the significant effects on the accumulation of mineral content (N, P, and K) in *Bougainvillea* plants. Ries et al. [27] also reported that TRIA application stimulates K^+, Ca^{2+}, and Mg^{2+} accumulation in tomato, maize, and cucumber seedlings by eliciting a secondary messenger, $L(^+)$ adenosine. Elevated mineral contents may stimulate plant growth and flowering in *Bougainvillea* plants. It could also be suggested that the increased K^+ content of the leaves may play a role in stomatal function.

Many investigators have explored the effects of TRIA on several basic metabolic processes, including photosynthesis, nutrient uptake, and enzymatic activities [8]. Photosynthetic capacity depends on photosynthetic pigments, such as chlorophylls *a* and *b*, and a salt-induced reduction in photosynthesis can be attributed to a decrease in chlorophyll content [28]. Here, it was reported that an accumulation of chlorophylls (*a*, *b*, and *a* + *b*) was significantly induced after foliar TRIA application. These results are similar to those reported by Muthuchelian et al. [25], who reported that TRIA treatment increased the synthesis of chlorophylls *a* and *b*, enhanced CO_2 assimilation, and increased both the starch, and sugars content in *E. variegata* seedlings under different stress conditions. The observation of changes in the carotenoid content in leaves following TRIA applications is supported by the finding of Moorthy and Kathiresan [29], who reported that TRIA treatments increased the leaf

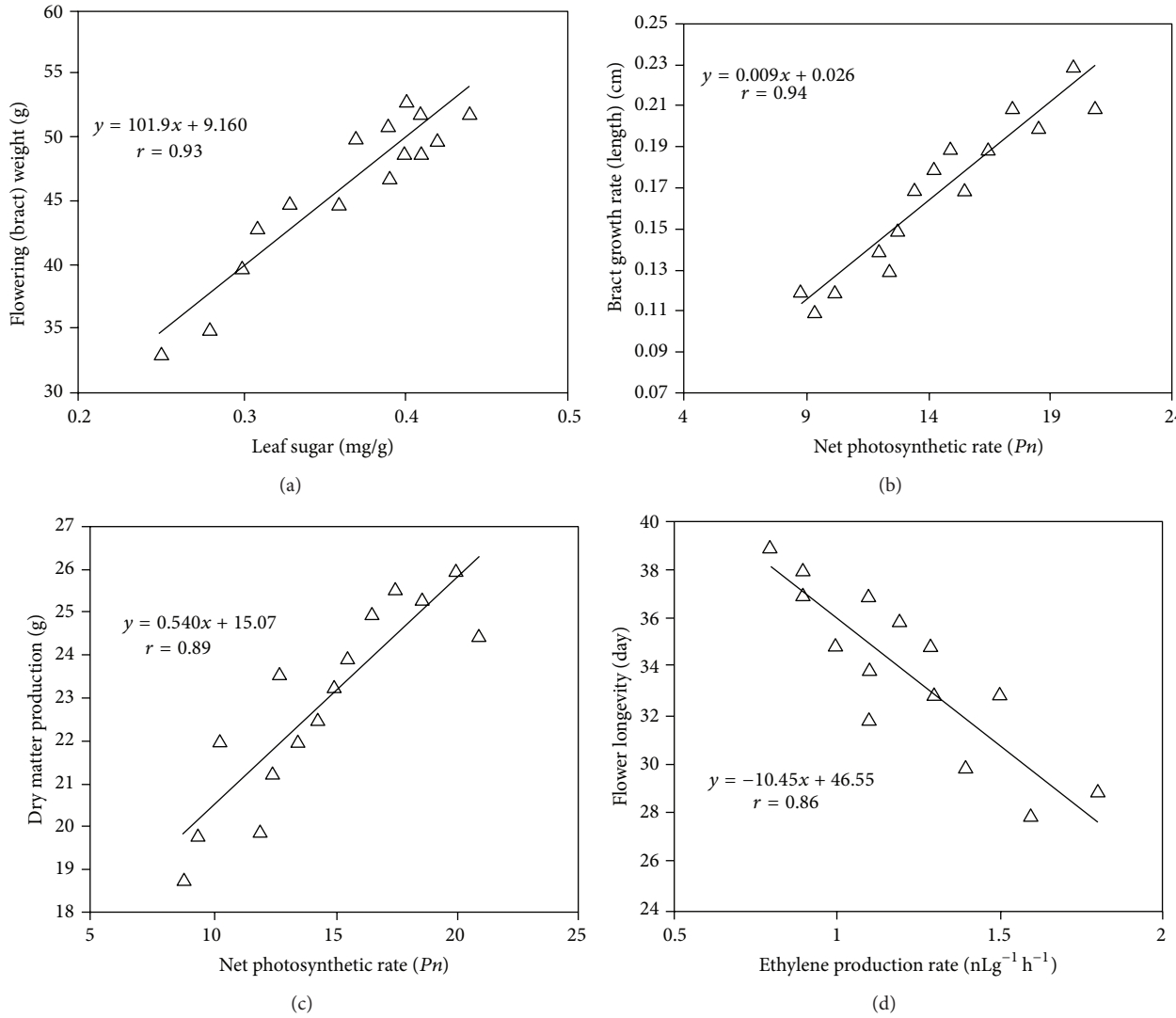

Figure 8: Regression lines for the correlations between flower weight (bract) and leaf sugar content (a), between net photosynthetic rate and bract growth rate (b), between net photosynthetic rate and dry matter production (c), and between flower longevity and the ethylene production rate (d) in TRIA-treated *Bougainvillea* plants under natural conditions.

carotenoid content of *Rhizophora apiculata*. The photosynthetic or quantum yield (Fv/Fm) of the treated plants was significantly affected by TRIA treatment in the present study. These quantum yield results are supported by the findings of Chen et al. [30], who stated that TRIA increased the minimal and maximal fluorescences (Fo and Fm) in rice plants. This effect might be due to TRIA increasing the antenna pigment level or the efficiency of excitation trapping at the active centers of PS-II.

TRIA has a stimulatory effect on photosynthesis, and the increased growth and dry weight of plants treated with TRIA have been attributed to an improvement in photosynthesis and an enhanced accumulation of photosynthates [31]. Chen et al. [9] reported that higher transcription of the rbcS gene was associated with the improved photosynthetic activity in TRIA-treated plants. These authors also illustrated that TRIA affected photosynthesis by increasing the level and activity of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) and by improving the status of the photosystems [31]. It has been demonstrated in a variety of plant species that the CO_2 fixation rate increases when TRIA is applied in nanomolar concentrations [32]. In the present study, it was found that treatment with TRIA increased the net photosynthesis and stomatal conductance of *Bougainvillea* plants. Stomatal conductance affects the photosynthetic rate by regulating CO_2 fixation in the leaf mesophyll tissue and is positively correlated with photosynthesis. Ivanov and Angelov [33] also reported that TRIA had positive effects on the net photosynthetic rate, stomatal conductance, and internal CO_2 concentration of pea plants. The changes in branch dry matter that we observed are in agreement with the findings of Muthuchelian et al. [25], who reported that TRIA application increased the fresh and dry biomass of *Erythrina variegata* plants. In this present study, it was also observed that plant growth rate and dry matter production were positively correlated with the photosynthesis rate in the

treated plants. A similar observation was reported by Eriksen et al. [31], who reported that dry matter production increased with the photosynthesis rate in maize.

The current study reported that TRIA treatments stimulated advanced flowering, reduced the node number required for the first flowering, and increased both the total flower number and the weight of flowers (bracts). These findings are supported by the results of Skogen et al. [10], who reported that TRIA treatment stimulates flowering, increases the growth and number of flowers, and improves the quality of chrysanthemum flowers. Ichimura et al. [34] reported that flower longevity was related to light intensity and the sugar supply to the sepals after flower opening. The present study demonstrated that TRIA treatment increased the flower longevity of *Bougainvillea* plants by delaying flower abscission. TRIA treatment may have increased the sugar supply to the leaf near the inflorescence and reduced the ethylene production by suppressing the activity of ACC synthase, thus increasing the longevity of the bracts and leaves. Moneruzzaman et al. [5] also reported that the sucrose application during postharvest storage increased the longevity of *Bougainvillea* bracts.

Ries [35] reported that TRIA resulted in simultaneous increases in soluble solids, soluble sugars and free amino acids in the treated plants. In this study, TRIA treatment also significantly increased the leaf TSS and sugar content of *Bougainvillea* plants. Houtz et al. [36] suggested that TRIA-stimulated an increase in the specific activity of the RuBisCO and phosphoenolpyruvate carboxylase enzymes. TRIA also increased the activity of a key respiratory enzyme, malate dehydrogenase, and other photosynthesis-related genes [37]. The elevated levels and activities of photosynthesis-related enzymes may have increased the TSS and sugar content of the leaves. The results also showed that the TSS content of leaves was positively correlated with advanced flowering and the increase in flower number of *Bougainvillea* plants. These findings are in agreement with those of Ramina et al. [38], who reported that higher TSS content of leaves stimulates flowering. In the current study, it was also observed that flower weight positively correlated with leaf sugar content in the TRIA-treated plants.

From this current study, it is clear that TRIA treatment produced a significant effect on both the phenol and flavonoid contents and the antioxidant activities of *Bougainvillea* plants. Similarly, Kumaravelu et al. [23] also reported that foliar TRIA application, at a dose of $0.5\,\mathrm{mg\,dm^{-3}}$, significantly elevated the saccharide, starch, amino acid, and phenol contents in green gram plants. TRIA may be responsible for the activation of PAL, chalcone synthase, and stilbene synthase gene expression. Grzegorczyk et al. [39] reported that TRIA treatment increased the biosynthesis of secondary metabolites and increased the antioxidant activities of *S. officinalis*. The results showed that the soluble protein content of *Bougainvillea* leaves increased significantly with TRIA treatment. These findings are similar to those of Chen et al. [9], who reported that TRIA application regulated ABA protein and downregulated the ABA gene in stressed rice plants. Kim et al. [40] also reported similar positive effects on the soluble protein, starch, sugar, and free amino acid contents in the leaves of *Oryza sativa* and *Zea mays*.

Enhanced sucrose phosphate synthase activity can be closely associated with an increase in sucrose accumulation in plant parts. The findings of this study demonstrated that TRIA application elevated the level and activity of the SPS enzyme during the vegetative and flowering growth stages of *Bougainvillea* plants. It was also observed that both the photosynthesis rate and SPS activity were positively correlated with TRIA treatment in *Bougainvillea* plants. This increasing rate of photosynthesis provides higher levels of phosphoglycerates (PGA), which in turn results in more fructose 6-phosphate and glucose 6-phosphate, increasing sucrose synthesis via SPS activity. The increase in sucrose accumulation may stimulate flowering, increase the number of flowers, enlarge the flower size, and delay abscission. Muthuchelian et al. [41] reported that the application of TRIA reduced the inhibition of PS-II and elevated the level and activity of RuBisCo in *E. variegate* plants under stress conditions. These authors also reported a TRIA-mediated maintenance in the photosynthetic machinery and a significant delay in the leaf senescence of water-stressed plants. In this study, it was also observed that TRIA significantly delayed leaf abscission in *Bougainvillea* plants. This effect may have been due to an increased accumulation of sugar in the treated leaves following TRIA treatment.

The ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase regulate ethylene synthesis and release in plants [42]. The constitutively synthesized ACC oxidase enzyme converts ACC to ethylene. Consequently, the accumulation of ACC augments ethylene production [43]. Here, we demonstrated that TRIA treatments led to a decline in ethylene production during both the vegetative shoot and flowering stages. The findings of this study are supported by the results of Saltveit and Dilley [44], who reported that TRIA in combination with either 10 mM kinetin or 10 μM benzyladenine in pea plant extract prevented the reduction in wound ethylene synthesis. The observed decline in ethylene production during both stages is most likely due to a downregulation of the 1-aminocyclopropane-1-carboxylic acid (ACC) gene by TRIA. Ethylene production increased in both the treated and untreated plants over time. However, the ethylene production rate was slower in the TRIA-treated plants than in the untreated plants. This effect is most likely due to the slower conversion of ACC to ethylene. It was reported in this study that bract and leaf abscission is higher in the control plants compared to the TRIA-treated plants, an effect that may be due to the higher rate of ethylene formation in the untreated plants. Our results also suggested that flower longevity is negatively correlated with ethylene production rate in treated plants.

5. Conclusion

The results have shown that the tested concentrations of TRIA used had a significant effect on photosynthesis, photosynthetic pigments, minerals, and biomass content of *Bougainvillea* plants. TRIA treatments also stimulated flowering and

increased the number and quality of flowers in addition to increasing protein, TSS, and sugar content, as well as the antioxidant and SPS activities in *Bougainvillea* leaves. Ethylene production was markedly reduced whilst the longevity of the leaves and bracts was increased in TRIA-treated plants. It can be concluded that treatment with 1.0 and 2.5 mg/L TRIA can enhance the physiological activities, flowering, and quality of potted *Bougainvillea* plants grown under natural conditions.

Acknowledgment

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Research Article

Use of Tissue Culture Techniques for Producing Virus-Free Plant in Garlic and Their Identification through Real-Time PCR

Hatıra Taşkın,[1] Gökhan Baktemur,[2] Mehmet Kurul,[3] and Saadet Büyükalaca[4]

[1] *Department of Plant Production and Technologies, Faculty of Agricultural Sciences and Technologies, University of Niğde, 51240 Niğde, Turkey*
[2] *Department of Biology, Faculty of Arts and Science, University of Osmaniye Korkut Ata, 80000 Osmaniye, Turkey*
[3] *Roche Diagnostics, 34394 Istanbul, Turkey*
[4] *Department of Horticulture, Faculty of Agriculture, University of Çukurova, 01330 Adana, Turkey*

Correspondence should be addressed to Hatıra Taşkın; hatirataskin1@gmail.com

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This study was performed for comparison of meristem culture technique with shoot tip culture technique for obtaining virus-free plant, comparison of micropropagation success of two different nutrient media, and determination of effectiveness of real-time PCR assay for the detection of viruses. Two different garlic species (*Allium sativum* and *Allium tuncelianum*) and two different nutrient media were used in this experiment. Results showed that Medium 2 was more successful compared to Medium 1 for both *A. tuncelianum* and *A. sativum* (Kastamonu garlic clone). *In vitro* plants obtained via meristem and shoot tip cultures were tested for determination of onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV) through real-time PCR assay. In garlic plants propagated via meristem culture, we could not detect any virus. OYDV and LYSV viruses were detected in plants obtained via shoot tip culture. OYDV virus was observed in amount of 80% and 73% of tested plants for *A. tuncelianum* and *A. sativum*, respectively. LYSV virus was found in amount of 67% of tested plants of *A. tuncelianum* and in amount of 87% of tested plants of *A. sativum* in this study.

1. Introduction

Total garlic production of Turkey was 105, 363 tons [1], and 18–20% of this production is provided from Taşköprü district of Kastamonu province. Kastamonu garlic clone (*A. sativum*) is suitable for winter consumption, can be stored for a long time, and is suitable for processing due to dry matter content [2]. *Allium tuncelianum*, called Tunceli garlic, is only common in Tunceli province of Turkey, especially around Munzur Mountains in Ovacık district, and it is known as an endemic to this region [3, 4]. Unlike other garlics with multiple-cloved bulb, *A. tuncelianum* has single-cloved bulbs and has small formations like a small bulbs, and it can also produce fertile flowers and seeds. The most important problem of garlic production in Turkey is infected areas due to pests and diseases carried via contaminated seedling. Garlic production areas and seedling production areas are not separated in Turkey. Pests and diseases can be carried via contaminated material in garlic propagated vegetatively because of sexual sterility. The most important ones of these diseases and pests are nematode (*Ditylenchus dipsaci*), white rot disease (*Sclerotium cepivorum*), and viruses that cause a loss of between 30% and 100% of production and the most common viruses are onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV). Fidan et al. [5] reported that these two viruses together affect the plant negatively and result in a yield loss of up to 78%. There is no effective chemical control method against viruses directly. For this reason, the most common method for obtaining virus-free plant is meristem culture technique. Different researchers [6, 7] have also reported that shoot tip remained insufficient for obtaining the virus-free plants. Biological (mechanical inoculation, indexing), serological (enzyme-linked immunosorbent assay—ELISA), and molecular (polymerase chain reaction—PCR) methods are used for detection of viruses in plants and identification of these viruses. Real-time 4 PCR assay, which is one of the PCR

types, is able to provide quantitative assessment. One of the main advantages of the real-time PCR is the very low chances of infection and detection of viruses with high efficiency. Detection of viruses through real-time PCR method has been performed successfully by many researchers such as Roberts et al. [8], Korimbocus et al. [9], Balaji et al. [10], Lunello et al. [11], Yılmaz [12], Mehle et al. [13, 14], and Fidan et al. [5].

The objectives of this study were (i) comparison of meristem culture technique with shoot tip culture technique to obtain virus-free plants, (ii) comparison of micropropagation success of two different nutrient media and two different garlic species (*A. tuncelianum* and *A. sativum*), (ii) to see the effectiveness of real-time PCR assay on detection of viruses.

2. Material and Methods

This study was conducted at the Department of Horticulture, University of Çukurova Turkey, and Adana Veterinary Control Institute, Turkey between the years 2009 and 2010. *Allium sativum* (Kastamonu garlic clone) and *Allium tuncelianum* garlic species determined to be infected with viruses were used as plant material. *A. sativum* and *A. tuncelianum* garlic samples were provided by Kastamonu-Taşköprü and Tunceli-Ovacık regions of Turkey, respectively.

2.1. Meristem and Shoot Tip Culture Studies. *A. sativum* and *A. tuncelianum* garlic species were propagated via meristem and shoot tip cultures. Cloves of garlic were waited in 25% sodium hypochlorite for 20 minutes for surface sterilization and washed 4-5 times with sterile water. Meristem and shoot tips from sterile cloves were extracted by sterile forceps and scalpels under a stereobinocular microscope in laminar flow and placed into glass tubes containing MS nutrient medium [15]. All cultures were incubated in the growth chamber at 25°C, under 3000 lux light and 8 hours dark and 16 h light photoperiod conditions. Germinated meristem and shoot tips were transferred to two different nutrient media (Medium 1: MS medium containing 0.5 mg L^{-1} 2-IP, 0.2 mg L^{-1} NAA, and 30 g L^{-1} sucrose; Medium 2: MS containing 2 mg L^{-1} BA, 0.5 mg L^{-1} IBA, and 30 g L^{-1} sucrose) for micropropagation. After 6 weeks, number of shoots per plant were recorded, and the shoots were subcultured. The experiment was designed in a completely randomized experimental design with three replications and thirty explants included per replication.

Variance analysis was conducted to evaluate the results, and Tukey test was used for controlling the significance of the differences.

2.2. Real-Time PCR Studies. Sixty *in vitro* plants (15 of them from meristem culture of *A. tuncelianum*, 15 of them from shoot tip culture of *A. tuncelianum*, 15 of them from meristem culture of *A. sativum*, and 15 of them from shoot tip culture of *A. sativum*) were used for real-time PCR studies. High Pure Viral Nucleid Acid Kit was used for RNA extraction of garlic samples. 200 μL working solution and 50 μL proteinase K were added to 1.5 mL nuclease-free microcentrifuge tube containing 100 mg plant samples and pulverized. After the mixture was mixed, it was incubated for 10 min at 72°C. Then, 100 μL binding buffer was added and mixed. Mixture was transferred into the upper reservoir of combination of high pure filter tube and the collection tube and spun for 1 min at 8,000 g in a centrifuge. Collection tube was removed and filter tube was placed into a new collection tube. Then 500 μL inhibitor removal buffer was added and spun for 1 min at 8,000 g in a centrifuge. Collection tube was changed with a new one. Then 500 μL wash buffer was added and spun for 1 min at 10,000 g in a centrifuge. The collecting tube was changed, and the washing step was repeated. Filter tube was placed into new sterile 1.5 mL nuclease-free microcentrifuge tube, and 75 μL elution buffer to elute the viral nucleic acid followed by spinning for 1 min at 13,000 g in a centrifuge. Filter tubes placed in the Eppendorf tubes were removed, and purified viral nucleic acid was extracted.

Obtained pure viral nucleic acids were converted to cDNA with reverse transcription PCR (RT-PCR) and incubated at −15/−20°C in a freezer. Transcriptor High Fidelity cDNA Synthesis Kit was used for converting RNA to cDNA. This process was completed in two steps. 2 μL random primer, 1 μL PCR-grade water, and 8.4 μL RNA per sample were used to prepare PCR mixture in the first step. This mixture was incubated at 65°C for 10 min. Prepared mixture in the second step consisted of 4 μL transcriptor reverse transcriptase reaction tampon, 0.5 μL protector RNAse inhibitor, 2 μL deoxynucleotide mix, 1 μL DTT, and 1.1 μL transcriptor reverse transcriptase enzyme. These two mixtures were mixed and incubated at 45–55°C for 10–30 min and then at 85°C for 5 min. Thereafter, cDNA synthesis was completed and stored at −20°C. Real-time PCR mix-conducted in a 20 μL volume containing 1xSYBR Green master mix (Roche applied science), 5 nM forward-reverse primers, and PCR grade water, using the following program: 10 min at 95°C for activation of DNA polymerase, 45 cycles of 10 s at 95°C, 30 s at 60°C, 1 s at 72°C, 30 min at 40°C and 30 s at 40°C for cooling in Roche LightCycler 2.0 Real-time PCR. Evaluation was performed by qualitative detection program of this thermocycler. Mixture containing 5 μL isolated DNA from samples, 2 μL SYBER Green master (it consisted, fast start Taq DNA polymerase, reaction buffer, $MgCl_2$, dNTP mix, and SYBER Green-Roche), 5 μL forward and reverse primers, 2.4 mM Mg, and PCR-grade water (total volume 20 μL) was prepared for the detection in real-time PCR using 600 s at 95°C for denaturation of DNA, 45 cycles of 10 s at 95°C, 30 s at 60°C, 2 min at 72°C, and 30 min at 40°C for cooling.

SYBER Green was used for detection of OYDV and LYSV viruses. The primers used in this study (Table 1) for OYDV and LYSV viruses were performed using primers registered in genbank [11] as forward OYDV primer (F-OYDV), 1099–1122 positions of sequences registered as AJ292223 in GenBank Database, as reverse OYDV primer (R-OYDV) 1306–1325 positions of AJ292223, as forward LYSV primer (F-LYSV) 789–808 positions of sequences registered as AY007693, and as reverse LYSV primer (R-LYSV) 839–854 positions of AY007693 [11].

Table 1: Primers used in the study [11].

Primer		bp
Forward-LYSV	5-TCTCGCACGGTATGCATTTG-3	20
Reverse-LYSV	5-GCCTCGCGCGCTCTAA-3	16
Forward-OYDV	5-AGTGATGCAGCTGAAGCATACATT-3	24
Reverse-OYDV	5-ACGTTACCATCCAGGCCAAA-3	20

3. Results

Ninety shoot tips and ninety meristems were used for *A. tuncelianum* in this experiment. Meristems transformed to plants in eight weeks. While 85 of meristems could grow without any problems, three of them could not germinate, and as infection problem was observed in two meristems. Cultured shoot tips were turned into plants in 3-4 weeks. Eighty-four shoot tips germinated healthy; only 1 shoot tip could not grow and 5 of ninety shoot tips were found to be infected. Plants obtained meristems of *A. tuncelianum* were transferred to two different nutrient media for micropropagation. Number of shoots were recorded in both first micropropagation and subculture were higher in Medium 2 compared to Medium 1, and the differences between the two media were found to be significant statistically. In the first propagation, 14.10 shoots/plant and 4.63 shoots/plant were obtained from Medium 2 and Medium 1, respectively. Similar results were provided from subculture. 13.27 shoots/plant in Medium 2 and 3.93 shoots/plant in Medium 1 were determined. Shoot tip culture results showed that Medium 2 was better compared to Medium 1. 11.37 shoots/plant and 2.41 shoots/plant were obtained from Medium 2 and Medium 1 in the first propagation, respectively. 8.93 shoots/plant and 2.36 shoots/plant were observed in Medium 2 and Medium 1 in subculture, respectively. Considered in terms of explants, meristem explants were found to be more successful compared to shoot tip explants in both garlic species and nutrient media.

The experiment for *A. sativum* was designated with 90 meristems and 90 shoot tips. While 89 meristems could transform into plant in meristem culture (the remaining one meristem was probably damaged), 87 shoot tips could germinate in shoot tip culture (the remaining 3 plants were infected). Meristem and shoot tip culture results of *A. sativum* showed that Medium 2 was more successful compared to Medium 1. Number of shoots/plant obtained from different tissue culture techniques and different garlic species are given in Table 2. As seen in Table 2, 11.67 shoots/plant and 5.41 shoots/plant were counted in Medium 2 and Medium 1 in the first micropropagation, respectively. In subculture, 11.61 shoots/plant and 5.56 shoots/plant were obtained on Medium 2 and Medium 1, respectively. These results compared with results of *A. tuncelianum*; number of shoots of *A. tuncelianum* in Medium 2 were higher than of *A. sativum* in both nutrient media. In Medium 1, *A. sativum* gave slightly higher results compared to *A. tuncelianum*. In shoot tip culture of *A. sativum*, Medium 2 and Medium 1 gave 9.84 shoots/plant and 2.93 shoots/plant for the first micropropagation, respectively. 10.17 shoots/plant from Medium 2 and 3.61 shoots/plant from Medium 1 were obtained in subculture.

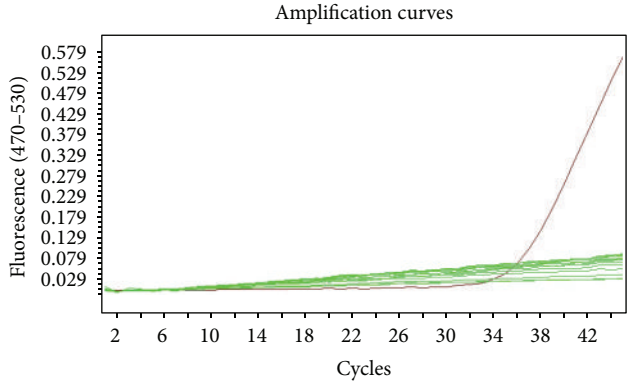

Figure 1: Application curves for meristem culture.

Sixty garlic plants propagated via meristem and shoot tip cultures were tested in terms of OYDV and LYSV viruses through real-time PCR method. Specific primer sequences and specific DNA fragments replicated with these sequences were detected with SYBER Green marking system. OYDV and LYSV viruses were not observed in plants obtained from meristem culture of both *A. tuncelianum* and *A. sativum*. Viruses were detected in shoot tip culture. OYDV virus was determined in 12 plants of *A. tuncelianum* and 11 plants of *A. sativum*. Ten plants of *A. tuncelianum* and 13 plants of *A. sativum* were found to be contaminated with LYSV virus. While OYDV virus was observed in the amount of 80% of tested plants and the amount of 73% of tested plants for *A. tuncelianum* and *A. sativum*, respectively, LYSV virus was found in the amount of 67% of tested plants of *A. tuncelianum* and the amount of 87% of tested plants of *A. sativum* in this study. Amplification curves of meristem and shoot tip cultures are given in Figures 1 and 2.

4. Discussion

Results of this research showed clearly that meristem culture technique was more effective compared to shoot tip culture technique in obtaining virus-free plant. All plantlets propagated by meristem culture technique were found completely clear in terms of OYDV and LYSV. Whereas virus problem could not be solved by shoot tip culture technique, viruses were detected in the majority of plantlets obtained by this technique. In meristem culture, only apical dome and a few leaf primordia are isolated and placed to nutrient media [16]. Tips of growing shoots (2 cm or less than 2 cm) are used in shoot tip culture. According to some researchers, there is a competition between cell proliferation, and the formation of the virus particles in meristem region of plant. Nucleic acid production capacity in meristematic tissue during cell division is used for cell division and this situation prevents the reproduction of virus. According to other researchers, transportation of viruses to the meristem region of the plant is prevented due to lack of transport system in meristem [17].

Table 2: Number of shoots/plant obtained from different tissue culture techniques on different garlic species.

Explant Type	First micropropagation (shoots/plant)			Sub-culture (shoots/plant)		
	Medium 1	Medium 2	LSD (5%)	Medium 1	Medium 2	LSD (5%)
Meristem culture (AT)	4.63[b]	14.10[a]	0.86	3.93[b]	13.27[a]	1.86
Shoot tip culture (AT)	2.41[b]	11.37[a]	1.73	2.36[b]	8.93[a]	2.16
Meristem culture (AS)	5.41[b]	11.67[a]	1.21	5.56[b]	11.61[a]	2.58
Shoot tip culture (AS)	2.98[b]	9.84[a]	1.73	3.61[b]	10.17[a]	2.16

AT: *A. tuncelianum*; AS: *A. sativum*.
[a,b]Means followed by different letters in the same column are significantly different regarding Tukey's multiple range test at $P < 0.05$.

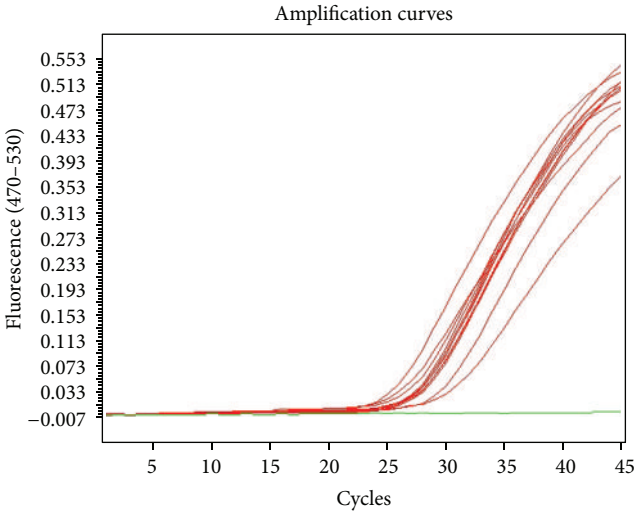

Figure 2: Application curves for shoot tip culture.

ELISA and real-time PCR techniques are used commonly in diagnostics of plant viruses. Real-time PCR method is more sensitive and specific than other techniques. Therefore, recently researchers prefer to use this technique alone or as comparison with ELISA. Many researchers have used real-time PCR method to identify viruses in different plants: for Plum Pox virus in plum by Varga and James [18], for OYDV and LYSV viruses in garlic by Lunello et al. [11], for zucchini yellow mosaic virus (ZYMV) in squash by Çalışkan [19], and for OYDV and SLV viruses in onion and garlic by Fidan et al. [5]. Real-time PCR method was found to be more sensitive than other methods in diagnosis of potato virus Y (PVY) by Mehle et al. [14]. Therefore, real-time PCR method was used in this study to detect viruses in garlic plants.

Also two different nutrient media were tested in terms of micropropagation success. Positive effect of BA and IBA on micropropagation of garlic was determined by Baktır [20]. NAA and 2-IP were used for *in vitro* propagation of garlic by Bhojwani [6]. Therefore, effects of these hormones on micropropagation of garlic were tested through using two different nutrient media in this study. Medium 2 containing 2 mg L^{-1} BA and 0.5 mg L^{-1} IBA was found to be more successful compared to Medium 1 containing 0.5 mg L^{-1} 2-IP and 0.2 mg L^{-1} NAA in this study.

One of the most important encountered problems in the production of garlic in Turkey is viruses. However, there is not any effective chemical application available against virus control. Meristem culture technique is widely used for obtaining virus-free plants. However, extracting the meristem regions of the plant is difficult, time-consuming and requires expertise. Therefore after achieving certain number of meristem, plants obtained from these meristems are multiplied using tissue culture techniques for commercial production. In this study, micropropagation capacity of *A. tuncelianum,* an endemic species of Turkey, and Kastamonu garlic clone of *A. sativum* were evaluated. The results obtained from the present study are very important for the scientist trying to develop virus-free plants and could also have significance of practical application which will in turn affect positively to increase yield with better quality of crop for the local farmers.

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Research Article

Adventitious Shoot Regeneration from Leaf Explant of Dwarf Hygro (*Hygrophila polysperma* (Roxb.) T. Anderson)

Mehmet Karataş, Muhammad Aasim, Ayşegül Çınar, and Muhammet Dogan

Department of Biology, Kamil Ozdag Faculty of Science, Karamanoglu Mehmetbey University, Yunus Emre Campus, 70200 Karaman, Turkey

Correspondence should be addressed to Muhammad Aasim; mshazim@gmail.com

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Dwarf hygro (*Hygrophila polysperma*) is an ornamental aquatic plant that changes its leaf colours to pinkish in high light. It is listed as a medicinal plant in medicinal plant lists of Indian states of West Bengal and Karnataka. It is also used as a screening tool for toxicities and a bioindicator to detect and control algae. The study reported *in vitro* adventitious shoot regeneration from leaf explants cultured on MS medium containing 0.10–1.60 mg/L Kin/TDZ with or without 0.10 mg/L IBA and 500 mg/L Amoklavin to eradicate endogenic bacterial contamination. Direct adventitious shoot regeneration started within one week from both culture mediums followed by late callus induction which was more prominent on TDZ containing media compared to Kin containing media. Addition of 0.10 mg/L IBA with both Kin and TDZ increased shoot regeneration frequency, mean number of shoots per explant, and mean shoot length. Maximum number of 16.33 and 20.55 shoots per explant was obtained on MS medium containing 0.80 + 0.10 mg/L Kin-IBA and 0.10 + 0.10 mg/L TDZ-IBA, respectively. Regenerated shoots were rooted on MS medium containing 0.20–1.00 mg/L IBA followed by successfull acclimatization in aquariums. Regenerated plantlets were also tested in jars containing distilled water that showed the pH 6–9 for the best plant growth and development.

1. Introduction

Aquatic plants are partially or completely water grown plants, and they are gaining popularity in traditional aquariums and water gardens [1]. In USA, water gardens are found in approximately 16 million houses [2]. In European countries, aquatic plants are also popular and are imported from abroad due to ever increasing popularity. Holland, France, Czech Republic, Germany, Hungary, Switzerland, Austria, Turkey, Latvia, and Estonia are the leading countries of Europe spending millions of Euros for the import of aquatic plants. The most imported aquatic plant is *Egeria densa* which is followed by *Cabomba caroliniana, Hygrophila polysperma, Vallisneria spiralis, Echinodorus bleheri, Vallisneria americana, Najas marina,* and *Hygrophila difformis* [3].

Hygrophila Polysperma (Roxb.) T. Anderson, commonly known as dwarf hygrophila, dwarf hygro, Miramar weed, or Indian weed, is an aquatic plant belonging to family the Acanthaceae. Dwarf Hygro is native to India and Malaysia and was introduced to USA states of Texas, Florida, and Virginia [4] and traded as Eastern Ludwigia in 1945 [5]. The plant is very popular aquatic ornamental plant [6] and became a part of aquariums all over the world. Dwarf hygro belongs to *Hygrophila* genus which contains almost 90 species; most of them are used for medicinal [7, 8] and antibacterial [9] purposes.

Dwarf hygro is an important ingredient of Ayurvedic system of medicine used for hemiplegia, stiff-neck, facial paralysis, and noise in the ears with headache [10]. The seeds of dwarf hygro are also used for treatment of other remedies in India [11], and the plant has been listed in the medicinal plant lists of Indian states of West Bengal [12] and Karnataka [13]. The research carried out in Sweden showed the efficient use of dwarf hygro to reduce the toxicity level [14]. Similarly, the plant has been used as bioindicator for algae control along with Indian ferns [15]. Very little is known about the importance of dwarf hygro micropropagation; therefore, the present study was designed to get adventitious shoot regeneration under *in vitro* conditions. Thereafter, the protocol can be employed to isolate secondary metabolites from the important medicinal plant.

Table 1: Effects of different Kin-IBA concentrations on shoot regeneration of *H. polysperma* from leaf explant.

Kin (mg/L)	IBA (mg/L)	Callus induction (%)	Shoot regeneration (%)	Shoots per explant	Shoot length (cm)
0.10	—	0.00^{b}	0.00^{c}	0.00^{e}	0.00^{c}
0.20	—	0.00^{b}	33.33^{b}	2.53^{d}	0.70^{ab}
0.40	—	0.00^{b}	50.00^{b}	1.83^{d}	0.47^{bc}
0.80	—	8.33^{b}	41.67^{b}	1.83^{d}	0.42^{bc}
1.60	—	8.33^{b}	41.67^{b}	1.33^{de}	0.45^{bc}
0.10	0.10	50.00^{a}	100.00^{a}	2.41^{d}	1.15^{a}
0.20	0.10	75.00^{a}	91.67^{a}	8.66^{c}	0.76^{ab}
0.40	0.10	75.00^{a}	91.67^{a}	11.20^{b}	0.78^{ab}
0.80	0.10	66.66^{a}	100.00^{a}	16.33^{a}	1.03^{ab}
1.60	0.10	75.00^{a}	91.67^{a}	12.58^{b}	0.82^{ab}

Means followed by different small letters within columns are significantly different using Duncan $P < 0.05$.

Table 2: Effects of different TDZ-IBA concentrations on shoot regeneration of *H. polysperma* from leaf explant.

TDZ (mg/L)	IBA (mg/L)	Callus induction (%)	Shoot regeneration (%)	Shoots per explant	Shoot length (cm)
0.10	—	75.00^{a}	87.50^{ab}	10.18^{bc}	0.43^{c}
0.20	—	93.75^{a}	100.00^{a}	12.68^{abc}	0.35^{c}
0.40	—	75.00^{a}	75.00^{bc}	11.95^{abc}	0.36^{c}
0.80	—	50.00^{b}	62.50^{c}	9.40^{c}	0.36^{c}
1.60	—	87.50^{a}	87.50^{ab}	13.67^{abc}	0.38^{c}
0.10	0.10	81.25^{a}	100.00^{a}	20.55^{a}	0.86^{a}
0.20	0.10	100.00^{a}	100.00^{a}	19.61^{a}	0.72^{b}
0.40	0.10	100.00^{a}	100.00^{a}	13.43^{abc}	0.38^{c}
0.80	0.10	100.00^{a}	100.00^{a}	18.43^{ab}	0.34^{c}
1.60	0.10	100.00^{a}	100.00^{a}	15.52^{abc}	0.31^{c}

Means followed by different small letters within columns are significantly different using Duncan $P < 0.05$.

2. Material and Methods

The *H. polysperma* plants were obtained from local aquarium traders of Karaman province of Turkey. The plants were confirmed by Professor Dr. Hasan Huseyin Atar of the Department of Fisheries of the Ankara University, Turkey. Four-five cm long twigs containing 5-6 nodes with attached leaves were first washed for 5 min under tap water. Thereafter, they were surface sterilized with 24% H_2O_2 (40% v/v) for 10 min followed by 3 × 5 min rinsing with sterilized distilled water by continuous stirring. The leaves were separated from twigs under sterile conditions, cultured on MS [16] medium for 2 weeks to obtain contamination free explants.

Leaf explants were cultured on MS medium supplemented with 3% sucrose and 0.10–1.60 mg/L Kin-0, 0.10 mg/L IBA (Table 1) or 0.10–1.60 mg/L TDZ-0, 0.10 mg/L IBA (Table 2) in Magenta GA^{7} vessels solidified with 0.65% agar. Culture media were also supplemented with 500 mg/L Amoklavin (antibiotic) to eradicate bacterial contamination, if any. Each experimental treatment was run in hexaplate and contained 8 explant (8 × 6 = 48 explants) with the pH of all media adjusted to 5.8 ± 0.1 before autoclaving (118 kPa atmospheric pressure, 120°C for 21 min). Both shoot and root regeneration experiments were repeated twice. All cultures were incubated under 16 h light photoperiod (5000 lux) using white Fluorescent lights.

The regenerated shoots were cultured on MS medium containing 0.10–1.00 mg/L IBA for rooting. After four weeks of culture, agar was removed carefully from the rooted plantlets without damaging the roots by washing under running tap water. Thereafter, the plants were transferred to aquariums containing tap water and sand. In another experiment, the plants were acclimatized in jars containing water at variable pH of 4.0–10.0 and then left open for for acclimatization in growth room at 23°C with 16 h light photoperiod for 3 weeks.

All data shown in percentages were subjected to arcsine transformation [17] before statistical analysis. Statistical analysis was performed as one way ANOVA using SPSS17 for Windows, and post hoc tests were performed using LSD or *t*-test.

3. Results

Leaf explants of *H. polysperma* were cultured on MS medium containing 0.10–1.60 mg/L Kin or TDZ with or without 0.10 mg/L IBA. Direct adventitious shoot regeneration without callus induction started from leaf tip on both Kin-IBA and

(a) (b) (c)

(d) (e)

Figure 1: *In vitro* adventitious shoot regeneration of *H. polysperma:* (a) shoot induction from leaf tip, (b) multiple shoot initiation, (c) callus induction, and ((d) and (e)) multiple induced shoots.

TDZ-IBA containing basal media. After one week of culture, visible shoot buds were noted on the leaf tips (Figure 1(a)) and the margins (Figure 1(b)) of the explants. It was followed by callus induction (Figure 1(c)) and induction of multiple shoots on MS medium containing Kin-IBA (Figure 1(d)) and TDZ-IBA (Figure 1(e)).

Callus induction (Figure 1(c)) started after 3-4 weeks of culture on both Kin-IBA and TDZ-IBA supplemented medium. However, callus induction from TDZ-IBA was earlier compared to Kin-IBA. Frequency of callus induction (%) on MS medium supplemented with Kin-IBA and TDZ-IBA ranged 0.00%–75.00% (Table 1) and 50.00%–100.00% (Table 2), respectively. Very low or marginal frequency of callus induction was recorded on Kin used singly. However, TDZ used singly induced more callus on leaf explants ranged 50.00%–93.75%. Addition of 0.10 mg/L IBA positively induced callus with both Kin and TDZ in the culture medium that ranged 50.00–75.00% (Table 1) and 81.25–100.00% (Table 2) on MS medium containing Kin-IBA and TDZ-IBA, respectively .

Comparing shoot regeneration frequency (%), it varied on both regeneration media with range of 0.00%–100.00% on MS medium containing Kin-IBA and 62.50%–100.00% on MS medium containing TDZ-IBA. Both Kin and TDZ without IBA induced lower shoot regeneration in ranges of 0.00%–50.00% and 62.50%–100.00%, respectively. However, inclusion of 0.10 mg/L IBA with Ki or TDZ was favorable and increased the shoot regeneration significantly to 91.67%–100.00% (Table 1) with Kin and 100.00% on all concentrations of TDZ (Table 2) .

Mean number of shoots per explants from MS medium supplemented with Kin singly yielded very low number of shoots (0.00–1.83) per explant (Table 1). Contrarily, TDZ singly induced more number of shoots per explant in range of 9.40–13.67 (Table 2). On the other hand, addition of 0.10 mg/L IBA with both Kin and TDZ positively increased the mean number of shoots per explant. Kin-IBA containing medium yielded 2.41–16.33 shoots per explants with maximum of 16.33 shoots obtained on 0.80 mg/L Kin-0.10 mg/L IBA (Table 1). Number of shoots per explant on TDZ-IBA containing MS medium ranged 13.43–20.55 per explant with maximum number of shoots induced on MS medium containing 0.10 mg/L TDZ-0.10 mg/L IBA (Table 2).

Relatively longer shoots were obtained on MS medium containing Kin-IBA compared to MS medium containing TDZ-IBA. Increase of both Kin and TDZ concentrations in the culture media alone or with 0.10 mg/L IBA hindered mean shoot length. However, addition of 0.10 mg/L IBA improved the mean shoot length when culture contained various concentrations of Kin and TDZ singly. Mean shoot length of Kin-IBA and TDZ-IBA containing media ranged 0.00–1.15 cm (Table 1) and 0.31–0.86 cm (Table 2), respectively. However, maximum shoot length on both growth variants in their own category was recorded on MS medium supplemented with 0.10 mg/L Kin/TDZ + 0.10 mg/L IBA.

Well-developed *in vitro* regenerated shoots were rooted on MS medium containing 0.10–1.00 mg/L IBA. 100.00% rooting was recorded after 4 weeks of culture. The rooted plants (Figure 2(a)) were successfully transferred to aquariums (Figure 2(b)) containing tap water and sand, where

Table 3: Effects of pH levels on plant height and number of internodes of *H. polysperma*.

pH	Plant height before the start of experiment (cm)	Plant height after 3 weeks (cm)	Change in plant height (%)	Number of internodes	Number of internodes after 3 weeks	Change in number of internodes (%)
4.0	3.75	4.12	9.86	3.50	4.75	35.71
5.0	3.37	4.25	26.11	3.00	4.75	58.33
6.0	2.75	4.25	54.54	2.25	4.50	100.00
7.0	2.87	5.00	74.21	2.75	4.75	72.72
8.0	3.37	5.12	51.92	2.75	4.75	72.72
9.0	3.62	6.00	65.74	2.75	5.25	90.90
10.0	3.37	4.87	44.51	3.25	4.25	30.76

Figure 2: Rooting and acclimatization of *in vitro* regenerated plants of *H. polysperma*: (a) rooted plantlets, (b) acclimatized plants in aquariums, and (c) plant acclimatization at different pH.

he plants achieved 100.00% survival and acclimatization after 2 months.

In vitro rooted plantlets were transferred to glass jars containing distilled water at pH of 4.0–10.0 (Table 3). The experiment was run in triplicate with 5 plants selected randomly and placed in the jar. The plant height and number of internodes of all plants were measured before transfer to jars. After three weeks of culture, the plants were reassessed for plant height and number of internodes. All plants in the experiment showed 100% survival at all pH

levels (Figures 2(c) and 2(d)) with visible effects on plant height and internodes After three weeks, an increase of 9.86%–74.21% (Table 3) in plant height was recorded, with minimum increase of 9.86% on cultures at pH 4.0 followed by 26.11% increase at pH 5.0. However, maximum increase of 74.21% in plant height was recorded at pH 7.0. On the other hand, minimum of 30.76% and 35.71% increase in number of internodes was recorded (Table 3) at pH 10.0 and 4.0, respectively.

4. Discussion

The study presents the first report of *in vitro* adventitious shoot regeneration from leaf explant of *H. polysperma.* Leaf explant has been used for adventitious shoot regeneration in other aquatic plants like *Nymphaea* [18], *Hygrophila auriculata* [19], *Rotala macrandra* [20], and *B. monnieri* [21]. Leaf explants responded well to both growth variants (Kin and TDZ). Direct adventitious shoot regeneration without callus induction was observed within two weeks from both culture medium used in the experiment. Direct shoot regeneration from leaf explant has been reported in *Spilanthes acmella* [22]. Shoot bud initiation from tips or edges showed the efficacy of leaf explant which might be due to presence of relatively younger and actively dividing cells in that zone. Valobra and James [23] also reported adventitious shoot regeneration from callus induced on leaf disc edges.

Callus induction by the use of cytokinins-auxins concentrations is a reported phenomenon, and callus induction from leaf explant has been reported in other aquatic plants like water lettuce [24] and *B. monnieri* [21]. Callus induction started late after 3-4 weeks of culture in this study. Likewise, late callus induction from seed explant has been reported by Aasim et al. [25] in hairy vetch and Javed et al. [26] in *Brassica napus.* It was also noted that growth variants in the culture medium were more supportive for shoot induction rather than callus induction. Results further showed that frequency of callus induction was affected by type and concentration of growth variants and presence or absence of IBA in the culture medium. In general, TDZ used singly induced more calluses compared to Kin used singly. TDZ results in callus induction better than other cytokinins [27]. TDZ-induced callus induction on different explants of many recalcitrant species as well as on medicinal plants has been reported [28]. Contrarily, Başalma et al. [29] reported suppression of callus formation by TDZ in *Astragalus cicer* hypocotyl and cotyledon explants. On the other hand, MS medium containing Kin or TDZ with IBA induced more calluses compared to MS medium containing Kin or TDZ without IBA. Similarly, Mirici [30] reported low callus formation from leaf explants in *Astragalus polemoniacus* cultured on a medium with TDZ used singly.

Results on shoot regeneration frequency also showed clear effects of type of growth variants. Comparing Kin and TDZ used singly, TDZ was more inductive compared to Kin. Vijayakumar et al. [31] reported 30.0%–95.0% and 50.0%–95.0% shoot regeneration frequency of *B. monnieri* cultured on BA and TDZ, respectively. On the other hand, both Kin and TDZ were very responsive in the presence of 0.10 mg/L IBA. Karatas et al. [21] reported 100.0% shoot regeneration on leaf explant of *B. monnieri* using various concentrations of BA-NAA. Contrarily, Aasim et al. [25] found insignificant effect of IBA on shoot regeneration frequency of hairy vetch.

Results on mean number of shoots per explants revealed the effects of growth regulator type, concentration and presence or absence of auxins in the mediums. Results showed that Kin used singly is not sufficient for shoot buds initiation with very low number of shoots per explant compared to TDZ used singly. Contrarily, Yenice [32] reported 57.82 and 50.74 plantlets per explant of *Lemna minor* on liquid MS medium containing with 0.05 mg/L Kin and 0.6 mg/L TDZ, respectively. However, provision of 0.10 mg/L IBA proved to be sufficient for multiple shoot regeneration. Combination of cytokinin + auxin irrespective of their concentrations and explant type has been reported for maximum number of shoots per explant in other aquatic plants. Öztürk [33] obtained maximum number of shoots per explants on apical meristem explant of ludwiga cultured on 0.05 mg/L TDZ + 0.1 mg/L NAA. Anthony et al. [34] also recorded maximum number of shoots from *Leucopogon verticillatus* on 10 μM TDZ + 5 μM IAA. Panigrahi et al. [19] recorded maximum number of shoots on MS medium containing with 2 mg/L BAP and 0.2 mg/L NAA in *Hygrophila auriculata.* Öztürk [35] recorded 80.56 shoots per explant from leaf explant of *H. difformis* cultured on MS medium containing 0.25 mg/L Kin and 1 mg/L NAA. Sumlu [20] reported 27.33 shoots per explant of *Rotala macrandra* cultured on liquid MS medium containing 0.25 mg/L BAP + 0.50 mg/L NAA.

Results on mean shoot length showed suppressive effects of TDZ compared to Kin used singly. Results further showed that increase in Kin/TDZ concentration resulted in stunted shoots; the results are in line with Lata et al. [36]. The suppressive effects of TDZ on shoot length might be consistent with its high cytokinin activity [37]. On the other hand, addition of IBA helped to overcome the negative effects of cytokinins; a has been reported in hairy vetch [25], fenugreek [38] and narbon vetch [39].

Regenerated shoots were successfully rooted using IBA in line with the findings of Tiwari et al. [40], Sharma et al. [41], and Karatas et al., [21]. Rooted plantlets were successfully acclamatized in aquariums which is an important step for *in vitro* regenerated plants and has been reported in other aquatic plants like *R. macrandra* [20], *L. repens* [33], *N. indica* [42], *A. sessilis* [43], *V. anagallis-aquatica* [44], *C. wendtii* and *C. beckettii* [45], and *B. monnieri* [21, 46].

In order to find out the most suitable pH level for acclimatization, the rooted plants were acclimatized directly under aquatic conditions at various pH levels. The results showed that a plant can survive at pH 4.0 to 10.0. The results are in line with the findings of Karatas et al. [21], who also reported no negative effects of pH levels and successfully acclimatized plants at pH 4.0–10.0. Results further showed that a plant showed slower growth at pH 4.0 and 10.0. and can grow vigorously at pH range of 6.0–9.0.

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Research Article

Protein Profiling and Histone Deacetylation Activities in Somaclonal Variants of Oil Palm (*Elaeis guineensis* Jacq.)

Jamilah Syafawati Yaacob,[1] Hwei-San Loh,[2] and Rosna Mat Taha[1]

[1] *Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *School of Biosciences, The University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor, Malaysia*

Correspondence should be addressed to Jamilah Syafawati Yaacob; jam_sya@yahoo.com

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Mantled fruits as a result of somaclonal variation are often observed from the oil palm plantlets regenerated via tissue culture. The mantling of fruits with finger-like and thick outer coating phenotypes significantly reduces the seed size and oil content, posing a threat to oil palm planters, and may jeopardize the economic growth of countries that depend particularly on oil palm plantation. The molecular aspects of the occurrence of somaclonal variations are yet to be known, possibly due to gene repression such as DNA methylation, histone methylation and histone deacetylation. Histone deacetylases (HDACs), involved in eukaryotic gene regulation by catalyzing the acetyl groups are removal from lysine residues on histone, hence transcriptionally repress gene expression. This paper described the total protein polymorphism profiles of somaclonal variants of oil palm and the effects of histone deacetylation on this phenomenon. Parallel to the different phenotypes, the protein polymorphism profiles of the mantled samples (leaves, fruits, and florets) and the phenotypically normal samples were proven to be different. Higher HDAC activity was found in mantled leaf samples than in the phenotypically normal leaf samples, leading to a preliminary conclusion that histone deacetylation suppressed gene expression and contributed to the development of somaclonal variants.

1. Introduction

Mantled fruits in oil palm (*Elaeis guineensis* Jacq.) are a result of somaclonal variation that is often observed when the oil palm plantlets are regenerated via tissue culture [1, 2]. The mantled phenotypes have finger-like fruits and a thick outer coating, hence reducing the seed size and also oil production significantly. The overall size of mantled fruits is generally smaller than the normal, in some cases without seed. The comparison between a phenotypically normal fruit and a mantled oil palm fruit is shown in Figure 1.

The fruit mantling phenomenon has also made the scaling-up process of oil palm clones to be difficult as about 5% of the clonal populations derived from tissue culture exhibits somaclonal variation phenomenon [3]. Those undesirable abnormal phenotypic differences include the development of abnormal flowers where the male parts of the flowers are "feminized" [4]. Specifically, in the case of abortive mantling phenomenon, no pollen is produced by the male inflorescences, and as for female inflorescences, a ring of supplementary carpels is produced surrounding the gynoecium, which in turn prevents the mantled oil palm fruits from ripening [5]. This mantling phenomenon poses a threat to oil palm planters and can further jeopardize the economic growth of countries that depend particularly on oil palm plantation. Therefore, the underlying factors that cause the formation of these somaclonal variants need to be investigated, so that a detection marker can be developed to serve as an early detection method for the mantled fruits. The current study aims to evaluate the involvement of histone deacetylase (HDAC) in the mantling phenomenon and hence brings us one step closer to producing an excellent detection marker at early vegetative stage of the seedling in the future.

Even though somaclonal variation is often reported as a result of tissue culture propagation, the occurrence of somaclonal variation may not be unique to in vitropropagation as it can happen naturally in somatic and reproductive tissues in plants [6], possibly triggered by genomic shock

Figure 1: Comparison between phenotypically normal (top) and mantled fruits (bottom). Source: from Advance Agricultural Resources Pty Ltd (AAR).

or plasticity. This happens when the plants have exhausted its usual physiological responses to environmental stress [7]. This therefore also explains why somaclonal variation is often produced in tissue culture, where the plants are unable to withstand tissue culture stress. However, there are also other external factors involved in inducing the production of these somaclonal variants, such as the departure from organized meristematic growth, the genetic makeup (genotype, ploidy) of the explant source, the use of plant growth regulators (type and concentration), and also the source of explants [8]. For example, in oil palm propagation via tissue culture, somaclonal variation may arise when flower tissues are used as the explant source [8].

The molecular aspects of the occurrence of somaclonal variation have not yet been fully investigated [1], but one of the most likely factors is gene repression. There are several factors that can result in gene repression such as DNA methylation, histone methylation, and histone deacetylation. Histone deacetylases (HDACs) involve in eukaryotic gene regulation by catalyzing the acetyl groups removal from the lysine residues on histone; hence, HDAC transcriptionally repress gene expression [9–13]. In histone acetylation, the ε-amino groups of lysines in the N-terminal domain of core histones are acetylated by histone acetyltransferases (HATs) with acetyl-CoA as the cosubstrate [14]; this type of modification is reverted back by the reaction of histone deacetylases (HDACs). Hence, it can be deduced that histone acetylation results in gene expression, whereas histone deacetylation yields the opposite outcome.

HDACs play the opposite role of HATs, whereby it is related to transcriptional repression and involved in gene silencing [15]. In plants, there are three families of HDAC, namely, the *RPD3/HAD* gene family, the *HD2* enzymes family (maize histone deacetylases) and the sirtuin family that is associated with yeast *SIR2* [15, 16]. The *SIR2* proteins are eukaryotic *NAD*+ dependent protein deacetylases that are involved in many important biological processes such as DNA repair, transcriptional modulation, and life span control [17]. Plants also have another HDAC type called the *HD2*-type deacetylases that is only unique to plants and is unrelated to the other three HDAC types [15]. HDAC often work together with DNA methyltransferases and *HMT*s in their action [15]. Examples on the effects of HDAC reaction include the experimental study of overexpression of rice HDAC1 that resulted in a boost to growth rate and a striking phenotypic change in rice [15]. In experiments conducted on *Arabidopsis*, mutations of the genes that encode for *Rpd3*-type HDAC *HDA6* showed that they were involved in gene silencing, while antisense inhibition of *HD2*-type HDAC leads to seed abortion [15]. There are also other examples on HDAC activity that have been observed in other plants, but all of them also imply that HDAC repress gene transcription and hence also repress gene expression [15].

Tian et al. (2005) suggested that histone acetylation and deacetylation reactions were actually reversible, promoter-dependent, and also locus specific, hence enabling an excellent control over gene regulation in response to developmental changes and environmental stimuli [11]. Therefore, due to the reversible nature of histone deacetylation process, this implies that the mantling phenomenon can be reversed over time, as shown by several oil palm trees [18]. However, the occurrence of somaclonal variation in oil palm would still cause a great loss, hence it is very important that the mantling phenomenon be detected at an earlier stage by using a detection marker. The present study aims to demonstrate the relationship of HDAC enzyme levels and protein profiles involved in the mantling phenomenon.

2. Materials and Methods

2.1. Sample Collection. Two categories of samples were used in this study, namely, the phenotypically normal fruits and the somaclonal variants (mantled fruits), where different parts of the trees were sampled: the leaves, fruits, and florets. All samples were collected from AAR (Applied Agricultural Resources Pty Ltd) oil palm plantation in Paloh Substation, Johor, Malaysia, with the help of AAR researchers (Advanced Agriecological Research). Six sample categories were studied including 100% abortive clonal mantled palm (AM), 50% fertile clonal mantled palm (FM), androgynous clonal palms (AD1 and AD2),and normal clonal palms (N1 and N2 and with 4 or more stigmas).

The mantling phenomenon can be visually observed at different levels, in terms of the number of "finger" present and the degrees of mantling (either 100% abortive mantled or 50% fertile mantled). Overall, the 100% abortive mantled fruits are generally smaller than the 50% fertile mantled fruits. This is because the 100% abortive mantled fruits would be aborted before they become mature, and therefore the collected fruits were smaller. In this study, only the "five-fingers" fruits were used in the protein extractions. The mantled fruits also have a different number of "finger," compared to one another although they might come from the same tree. Some of them may have four, five, or even six "finger", as shown in Figure 2.

Figure 2: Different degree of mantling (number of "finger"). Source: from AAR.

2.2. DNA Extraction and SSR Analysis. Frozen leaves (2 g) of clonal lines of oil palm trees were ground to powder form using a mortar and liquid nitrogen. Modified CTAB method was employed in DNA extraction experiments, whereby PVP-40, ascorbic acid, DIECA, and 2-mercaptoethanol were added to the extraction solvent. The extracted DNA was subjected to SSR analysis by using 9 degenerate primers [19] to prove their clonal origin.

2.3. Total and Nuclear Protein Extraction. 60 mg of frozen leaf, fruit (mantled and normal), and floret samples was ground to a fine powder using a mortar and liquid nitrogen. Total protein extraction from the tissue samples was done using "Plant Total Protein Extraction Kit" (Sigma-Aldrich) and subjected to protein concentration assays (Pierce 660 nm Protein Assay (Thermo Scientific)), followed by subsequent SDS-PAGE analysis to allow for the visualization of their protein profiles. Nuclear protein extraction was also carried out from leaf tissues (20 g) using "Plant Nuclei Isolation/Extraction Kit (CelLytic PN)" (Sigma-Aldrich).

2.4. HDAC Analysis and ELISA Assay. The extracted nuclear protein extracts (900 mg) were subjected to HDAC activity assay using EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek). In this part of the research, the histone deacetylase enzyme activity was measured by means of enzyme-linked immunosorbent assay (ELISA) at 450 nm. The HDAC level of all sample categories were compared.

2.5. Statistical Analysis. Assessment of results was conducted using randomized complete block design (RCBD) with 3 replicates and statistically analyzed using analysis of variance (ANOVA), whereby mean comparisons were done using Duncan's multiple range test (DMRT) with the least significant differences at 5% level.

3. Results and Discussion

3.1. DNA Extraction and SSR Analysis. Two out of nine primers (primer P1T6 and P4T10) gave good results verifying that all samples were clonal siblings, as shown in Figures 3 and 4.

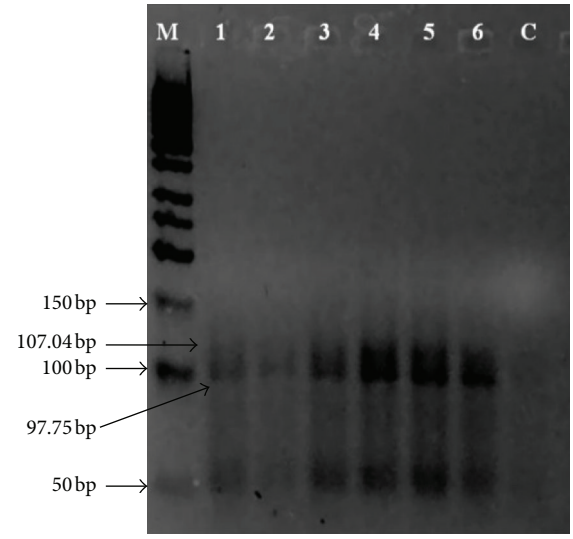

Figure 3: Amplification profile of SSR primer P1T6 (1: AM, 2: FM, 3: AD1, 4: AD2, 5: N1, 6: N2, M: Fermentas GeneRuler 50 bp DNA ladder, and C: control).

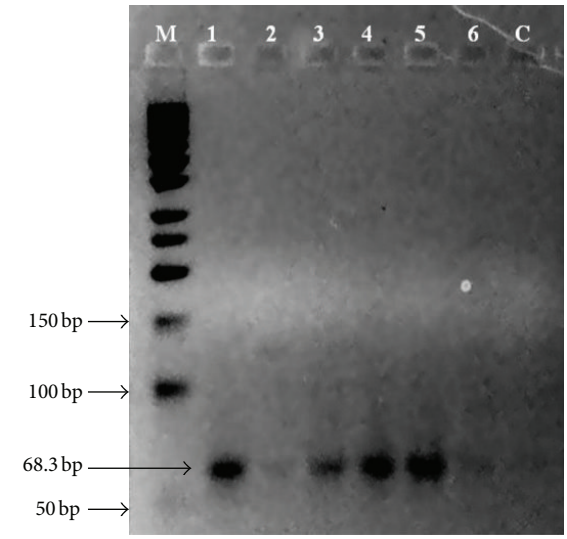

Figure 4: Amplification profile of SSR primer P4T10 (1: AM, 2: FM, 3: AD1, 4: AD2, 5: N1, 6: N2, M: Fermentas GeneRuler 50 bp DNA ladder, and C: control).

3.2. Total Protein Profiling and HDAC Analysis. Figures 5, 6, and 7 show the total protein profiles of the leaves, fruits, and florets, respectively as electrophoresed in SDS-PAGE gels. The electrophoresed protein fragments from the leaves were similar in all three samples (100% abortive mantled, 50% fertile mantled, and phenotypically normal). However, some of the electrophoresed protein fragments from the fruit and floret samples of 100% abortive mantled and 50% fertile mantled were different from those of the phenotypically normal.

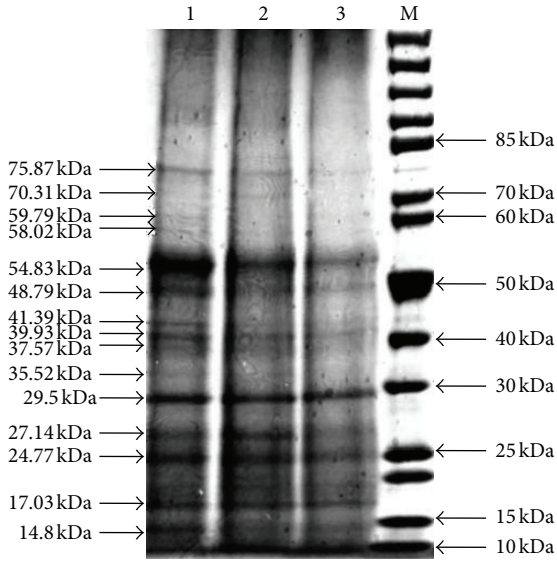

Figure 5: Total protein profiles of leave (1: phenotypically normal, 2: 50% mantled, 3: 100% mantled, and M: PageRuler Unstained Protein Ladder).

As observed in Figure 6, there was one prominent band that was present in all three fruit samples, which was about 55.12 kDa in size. Other than that, the phenotypically normal fruit also had two other unique prominent bands present, which were about 28.31 kDa and 18.77 kDa in size, while both the 100% and 50% abortive mantled fruits also had another one prominent band, which was about 26.06 kDa in size. This 26.06 kDa protein band was also present in the total protein profile of the phenotypically normal fruit, but that band was much fainter than that of the 100% and 50% abortive mantled fruits.

More interestingly, both the 100% and 50% abortive mantled fruits had four protein bands specifically unique for both of the samples, and that particular protein bands were not present in the phenotypically normal fruit protein profile. Those four bands were about 28.53 kDa, 27.89 kDa, 24.65 kDa, and 17.59 kDa in size. These findings might be due to the upregulation on certain amino acid synthesis, which should not usually occur, like in the phenotypically normal fruit [20, 21]. Besides that, there were also seven protein bands that were unique in the protein profile of the phenotypically normal fruit but were completely absent from the protein profile of the mantled fruits. Those seven bands were about 48.35 kDa, 39.55 kDa, 28.31 kDa, 24.71 kDa, 20.34 kDa, 18.77 kDa, and 14.68 kDa in size.

As for the florets, the banding patterns for all the samples were similar, except for two particular bands that were present in the phenotypically normal floret sample but absent from both the 100% mantled and 50% mantled florets (Figure 7). Those two bands were about 99.81 kDa and 84.86 kDa in size. The banding patterns of the protein polymorphism profiles of all three categories were summarized in Table 1.

The average HDAC activity levels of both 100% and 50% mantled samples were significantly higher than the HDAC activity level of the phenotypically normal sample, where 1030.869 ng/mL and 1173.888 ng/mL of average HDAC activity levels were recorded for 100% and 50% mantled samples respectively, while 614.557 ng/mL of average HDAC activity level was recorded for the phenotypically normal sample (Figure 8).

The mantling phenomenon undergoes an epigenetic regulation with similar underlying genomic sequences in all kinds of plant tissues; changes are produced at the gene expression level. This study aimed to investigate the involvement of HDAC enzyme in fruit mantling phenomenon, whereby the target was the chromatin (DNA and histones) inside the nucleus. The chromatin content of the cells would be similar despite the different tissues of leaves, florets and fruits. It is of greater interest and preference to determine the somaclonal variations at earlier time especially during the vegetative stage, rather than the reproductive stage after 4-5 years of growth, which latter has wasted time, manpower, and money. With the general aim of development of an early detection biomarker and the availability of more straightforward nuclear protein extraction method from the leaves, the HDAC activity assay was conducted on the leaf samples.

Out of the total 9 primers [19] used in the SSR analyses, two primers (primer P1T6 and P4T10) gave good results, whereas some of the other primers did not even produce any amplification, possibly due to the fact that those primers were degenerate primers [19]; hence, although they did work on the oil palm leaf samples in the previous study, they might not work on oil palm with slightly different genomes. As observed from Figures 3 and 4, all of the samples produced bands of similar size (~50 bp and ~100 bp in size for Primer P1T6 and about 63.5 bp in size for Primer P4T10), but with different degree of band intensities. Hence, it can be deduced that all of the six trees sampled in the study were of the same clonal origin and genotype.

The different total protein polymorphism profiles attributing to mantling morphologies with different severity (50% versus 100% mantled) were successfully shown for the leaves, fruits, and florets of the 100% mantled, 50% mantled, and phenotypically normal trees. As observed in Table 1, all three leaf samples produced similar banding patterns (Figure 5) although some of the bands appeared fainter than the comparable bands from the other samples. However, in contrast to the leaf total protein profile, the banding patterns of both the 100% mantled and 50% mantled fruits and florets (Figures 6 and 7) were similar, but both of them had different banding patterns as compared to the phenotypically normal fruits and florets, as clearly depicted in Table 1. This might be due to housekeeping genes inside the fruits and florets that may have been upregulating and downregulating the synthesis of certain proteins [20, 21], and hence causing the proteins that should have been present in a phenotypically normal fruits and florets to be absent in mantled fruit and floret samples instead. This may also cause the synthesis of new proteins in the mantled tree that were not present in a phenotypically normal tree.

Table 1: Estimated molecular size of protein fragments of 100% abortive mantled, 50% fertile mantled, and phenotypically normal samples (leaf, fruit, and floret).

Leaf			Fruit			Floret		
AM (kDa)	FM (kDa)	N (kDa)	AM (kDa)	FM (kDa)	N (kDa)	AM (kDa)	FM (kDa)	N (kDa)
								99.81
								84.86
75.87	75.87	75.87						
70.31	70.31	70.31						
59.79	59.79	59.79						
58.02	58.02	58.02						
						56.82	56.82	56.82
			55.12	55.12	55.12			
54.83	54.83	54.83						
48.79	48.79	48.79						
					48.35			
						44.54	44.54	44.54
						41.79	41.79	41.79
41.39	41.39	41.39						
39.93	39.93	39.93						
					39.55			
37.57	37.57	37.57						
35.52	35.52	35.52						
			32.15	32.15	32.15			
						31.47	31.47	31.47
29.50	29.50	29.50						
			28.53	28.53				
					28.31			
						28.21	28.21	28.21
			27.89	27.89				
27.14	27.14	27.14						
			26.06	26.06	26.06			
						25.12	25.12	25.12
24.77	24.77	24.77						
					24.71			
			24.65	24.65				
					20.34			
					18.77			
			17.59	17.59				
17.03	17.03	17.03						
						16.28	16.28	16.28
14.80	14.80	14.80						
					14.68			
						10.52	10.52	10.52

AM: 100% abortive mantled, FM: 50% fertile mantled, and N: phenotypically normal.

More importantly, the alteration of the proteins was targeted in the fruits but not in the leaves. Although those seven and those two protein bands were present in the phenotypically normal fruits and florets (Table 1), respectively, their absence in the mantled fruits and florets indicated that the mantling phenomenon occurs in the absence of certain proteins resulting from gene repression during protein synthesis which correlated with the findings by Tian et al. (2005) in their study on *Arabidopsis thaliana* [11]. These protein polymorphism profiles indicated that the mantling phenomenon was tissue specific and occurrence of somaclonal variations had started as early as at the floret developmental stage. This could lead to the speculation that the variations might even be detected during floral initiation and floral organ developmental stages. However, further investigations will be conducted in order to prove this. The

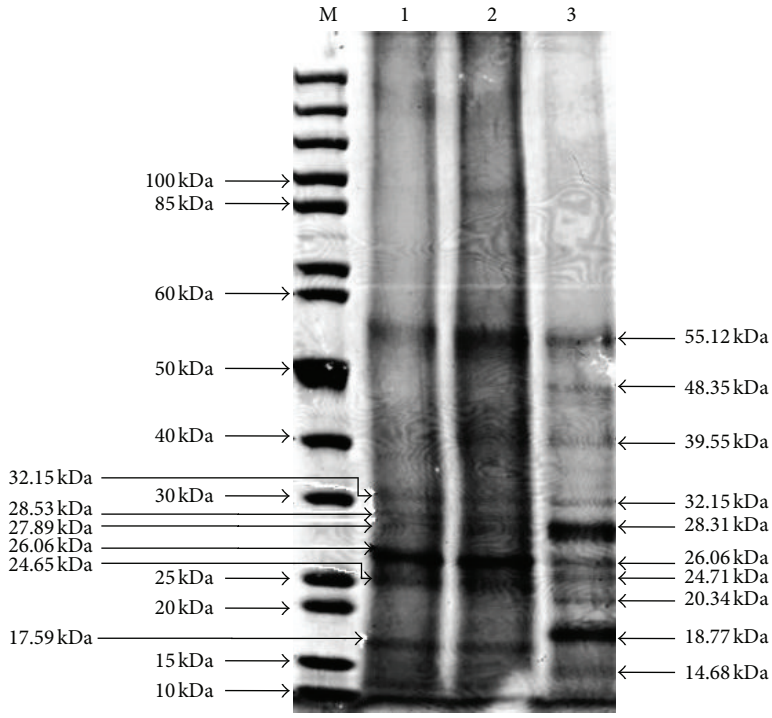

Figure 6: Total protein profile of fruits (1: 100% mantled, 2: 50% mantled, 3: phenotypically normal, and M: PageRuler Unstained Protein Ladder).

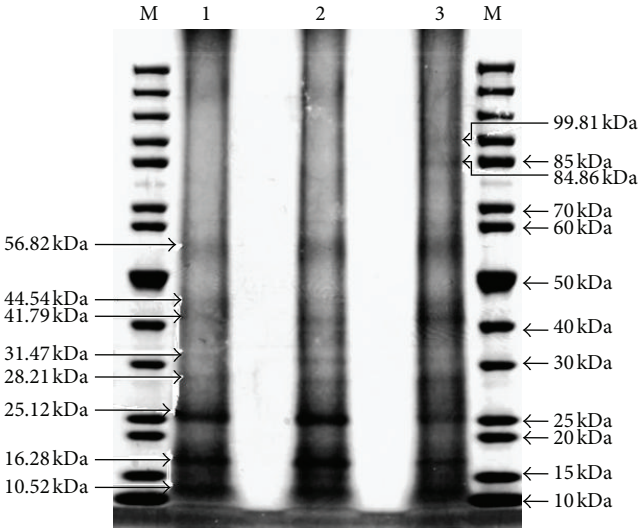

Figure 7: Total protein profile of florets (1: 100% mantled, 2: 50% mantled, 3: Phenotypically normal, and M: PageRuler Unstained Protein Ladder).

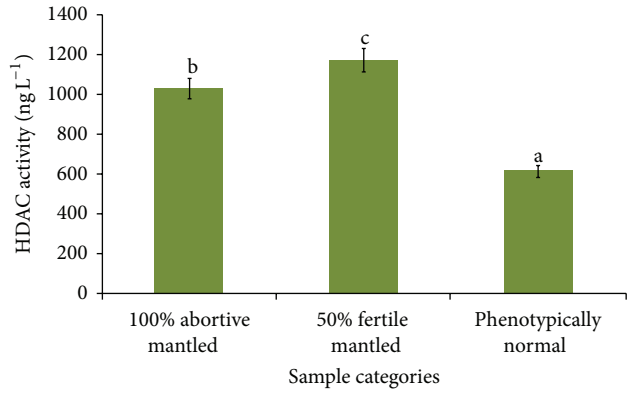

Figure 8: Average HDAC activity levels of leaf samples. Mean values with different letters are significantly different at $P < 0.05$.

differences of the protein profiles were very valuable for further investigation on the specific proteins involved in the mantling phenomenon, from which the identity of the different protein fragments present could be established and hence serve as a guideline to investigate the developmental stage and protein synthesis pathway involved in the mantling phenomenon.

Based on the findings of this study, it was suggested that histone deacetylation was causing this phenomenon, where the enzyme histone deacetylase (HDAC) had been involved in eukaryotic gene regulation by catalyzing the acetyl groups removal from the lysine residues on histone, and hence transcriptionally repressed gene expression [22]. This therefore disturbed the normal transcription and translation processes involved in the different developmental pathways which should have occurred (like in the phenotypically normal fruits), therefore causing certain amino acids (which would be normally produced) not to be produced, while some other amino acids which should be absent in the phenotypically normal fruits to be synthesized.

This hypothesis was supported by the different HDAC activity shown by the nuclear proteins of mantled and phenotypically normal leaves. As shown in Figure 8, theHDAC activity levels of both the 100% abortive mantled and 50% fertile mantled were significantly higher than that of the phenotypically normal leaves. Hence, it could be deduced that the HDAC enzyme had caused a certain extent of gene repression in the mantled samples because it works oppositely with the HAT, which had contributed to the acetylation and switching on the gene(s) to be expressed. However, the HDAC activity level of the 50% fertile mantled was slightly higher than that of the 100% abortive mantled. This might be due to the inclusion of the nonhistone proteins in the nucleus for the function of HDAC [23]. Therefore, as we studied the HDAC enzyme activity as a whole but not the mantling expression-specific one, there were some non-histone protein activities concurrently taking place and had contributed to the elevated HDAC level. This might have induced a higher level of HDAC activity in the 50% fertile mantled than the 100% abortive mantled. Besides, a different site of lysine deacetylation might contribute to the gene repression effects. Nevertheless, histone deacetylation is jeopardizing the normal protein synthesis that should have occurred, thus causing certain proteins not to be synthesized [15]. The disruption of the normal development process should have caused the mantling abnormalities to occur, whereby it could be speculated that the absence of the seven (fruits) and two (florets) protein bands from the mantled fruits and florets might be the result of histone deacetylation, which had caused the expression of these proteins' coding genes to be switched off.

Generally, this study had facilitated the establishment of protein polymorphisms between oil palm somaclonal variants, which could serve as a principle protocol for further investigations in proteomics. More experiments such as different protein extraction/fractionation methods and two-dimensional SDS-PAGE will be conducted on more oil palm samples in the future in order to further verify the protein polymorphisms as mentioned in this paper. Besides, the protein bands of interest could be excised from the gel and digested for mass spectrometric analysis for their characteristics and identities.

4. Conclusions

In this study, the different protein polymorphism profiles of the somaclonal variants involved in the mantling phenomenon have been clearly elucidated. Based on the HDAC activity assay, it was shown that histone deacetylation did involve in the fruit mantling phenomenon.

Acknowledgments

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Research Article

Effects of IAA, IBA, NAA, and GA3 on Rooting and Morphological Features of *Melissa officinalis* L. Stem Cuttings

Hakan Sevik[1] and Kerim Guney[2]

[1] *Landscape Architecture Department, Faculty of Engineering and Architecture, Kastamonu University, 37150 Kastamonu, Turkey*
[2] *Forest Engineering Department, Forestry Faculty, Kastamonu University, 37150 Kastamonu, Turkey*

Correspondence should be addressed to Hakan Sevik; hakansevik@gmail.com

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This study analyzed the potential of producing *Melissa officinalis* L. using stem cuttings. Four different hormones (IAA, IBA, NAA, and GA3) were applied to the cuttings, with and without buds, in two doses (1000 mg/L and 5000 mg/L), and after 60 days, 10 morphological characteristics of newly generated plants were detected, and a statistical analysis was carried out. The results of the study show that the cuttings with at least one bud must be used in order to produce *M. officinalis* using stem cuttings. Even though the auxin group hormones (IAA, IBA, and NAA) do not have an apparent effect on rooting percentage, these hormones were detected to affect the morphological characteristics of the newly generated plants, especially root generation. GA3 application has a considerable effect on stem height.

1. Introduction

Lemon balm (*Melissa officinalis* L.) belongs to the family Lamiaceae and grows widely in central and southern Europe and in Asia minor [1]. It is also found in tropical countries (Brazil) where it is popularly known as erva-cidreira and *Melissa* [2]. It is an aromatic (lemony) perennial herb, up to about 1 m high. Parts mostly used are dried leaves, often having flowering tops [3].

Green lemon aromatized leaves of this plant are used as fresh leaves, as well as in their dried form in salads, sauces, soups, with vegetables and meat, and in desserts. This plant is used in brewing certain alcoholic drinks and liquors and is consumed as an herbal tea. It is also used as an ornamental plant and as a border plant, particularly in gardens [4].

M. officinalis has been used in a variety of practical applications in medical science. The leaves contain volatile oils [5]. The leaf also contains polyphenolic compounds: caffeic acid derivatives in large proportions, such as rosmarinic acid, trimeric compounds, and some flavonoids [6].

M. officinalis can modulate a number of behavioral measures, with indications including administration as a mild sedative, in disturbed sleep, and in the attenuation of the symptoms of nervous disorders, including the reduction of excitability, anxiety, and stress [7]. *M. officinalis* extracts can attenuate the subjective effects of laboratory-induced stress [8]. It can be a useful herbal medicine for the treatment of gastrointestinal spasms [9]. *M. officinalis* L. has an antispasmodic and antimeteoric activity, as previously shown in animals and humans [10]. Schnitzler et al. [11] reported that *M. officinalis* oil might be suitable for topical treatment of herpetic infections. It has acetylcholine receptor activity in the central nervous system, with both nicotinic and muscarinic binding properties [12].

M. officinalis is predominantly sold in combination with other herbs, with, as an illustration, 49 products containing lemon balm in the German pharmaceutical industry's current "Rote Liste" (2001) drug catalogue [13]. From its Moorish introduction into Spain in the seventh century, its cultivation and use spread throughout Europe by the middle ages [13]. It is cultivated throughout the world because of its culinary properties [1].

Due to its economic importance, this plant is produced in large fields in several European countries such as France,

Germany, Bulgaria, and Romania as well as in North America. Even though the lemon balm plant naturally spreads on the flora of our country, its agriculture level is not satisfactory, and occasionally it is collected from nature and exported [14].

A significant amount of the lemon balm that is widely used in Turkey is collected from the natural flora. This includes cultivation of lemon balm, which has a very high economic importance and will prevent the excessive and senseless destruction of natural flora, at least to a certain extent. In this study, the potential of producing *M. officinalis* L. using stem cuttings was analyzed. For this purpose, four different hormones were applied to the stem cuttings in two doses, and the effect of nine applications, together with the control group, on 10 morphological characteristics was analyzed.

2. Materials and Methods

M. officinalis cuttings used in this research were collected from the Daday district of the province of Kastamonu in Turkey. The cuttings were collected on September 8, moisturized, and stored in germination turf. The applications on the cuttings brought to the laboratory were carried out on September 9, as explained hereinafter.

(a) Two-thirds of the 3 × 3 × 15 cm polyethylene tubes were filled with Klasmann germination turf in the laboratory.

(b) The cuttings were cut to 2.5 cm long pieces using sterile lancets and grouped as cuttings with and without buds.

(c) Solutions of four different hormones in two different doses (1000 mg/L and 5000 mg/L) were applied to the cuttings, respectively, and nine application groups were created, consisting of eight hormone application groups and a control group. The applications were carried out by imbruing the cuttings in hormone for 4 to 5 minutes. This application was composed of 3 replications and 15 cuttings in each replicate (15 cuttings with buds, and 15 cuttings without buds). Five cuttings were placed in each tube without any contact, covered with approximately 2 cm germination turf, and moisturized immediately.

(d) These tubes were placed in boxes with holes at room temperature (20–25°C), did not receive direct sun light, and were watered two times a day for 60 days. As the turf reached the saturation point, no water accumulation was generated, as the tubes and the boxes the tubes were placed in contained holes, and the surplus water was released.

(e) Measurements were carried out on November 8, which is the 60th day of the procedure. The turf in the tubes was poured on a laboratory bench, the roots were cautiously cleaned, and the number of the roots was defined. The average length of the roots was measured using a digital microcompass. After this procedure, stems and leaves were measured. All of these measurements were carried out using a digital microcompass with 0.01 mm precision, and the results were entered in a table. Ten morphological characteristics for each cutting were defined at the end of the study, including rooting percentage (RP), stem length (SL), stem length without branch (SLB), diameter (SD), number of leaves (LN), length of leaves (LL), size of leaf blade (LS), width of leaf blade (LW), number of roots (RN), and length of root (RL).

Variance analysis was applied on the data, using the SPSS 17.0 package program. The Duncan test was applied for the characteristics with at least 95% level of statistical reliance, and as a result, homogenous groups were acquired and interpreted.

3. Results

At the end of the study, no new stem was formed in the stem cuttings without buds. This result implies that the cuttings with buds must be used while producing *Melisa officinalis* with stem cuttings. Hormone applications in different doses affect the germination percentages, as well as the characteristics of the germinated individuals, at different levels. The data acquired with the results of the study, the results of variance analysis applied on these data, and the Duncan test are given in Table 1.

When the values stated in the table are analyzed, no germination is observed in the IAA hormone at a 5000 mg/L dose. The highest germination percentage values were acquired with 1000 mg/L IAA and 1000 mg/L GA3 hormone applications. The values acquired as a result of these applications are higher than the germination values acquired in the control group; however, according to the results of the Duncan test, these values are in the same homogenous group with the control group. According to the results of the Duncan test, the first homogenous group is composed of the application in which no germination was observed; 1000 mg/L IAA and 1000 mg/L GA3 applications together with the control group are included only in the second homogenous group, and the other applications were included in both homogenous groups.

According to the values stated in the table, significant differences with a 95% statistical reliance level arose between the applications; however, the statistical reliance level of the differences arose in accordance with the other characteristics was 99.9%. This result indicates that even though the hormone applications did not reveal the expected effect on germination percentage, they have a considerable effect on other characteristics.

When the values stated in the table are analyzed, it is observed that the longest seedlings with 81.72 stem length without branches and 96.75 total stem length were produced with the 5000 mg/L GA3 application. The seedlings produced in the control group have 55.57 total length and 30.12 stem length without branches. In this case, the length of the 5000 mg/L GA3 seedlings is 2.71 times greater than the control group in terms of stem length without branches and 74% greater in total length. Similarly, the length of 1000 mg/L GA3

Table 1: Mean values of characters, results of variance analysis, and the Duncan test.

Hormone	Dose	RP	SL	SLB	SD	LN	LL	LS	LW	RN	RL
IAA	1000	$44{,}0^{b}$	$41{,}55^{a}$	$16{,}56^{a}$	$1{,}03^{b}$	$5{,}00^{b}$	$21{,}55^{de}$	$12{,}27^{abc}$	$16{,}45^{cd}$	$3{,}0^{bc}$	$10{,}74^{ab}$
IAA	5000	0^{a}									
IBA	1000	$17{,}2^{ab}$	$60{,}71^{bc}$	$37{,}46^{bc}$	$1{,}07^{b}$	$6{,}00^{b}$	$10{,}02^{a}$	$20{,}49^{e}$	$19{,}27^{d}$	$4{,}0^{d}$	$54{,}02^{e}$
IBA	5000	$8{,}0^{ab}$	$52{,}83^{ab}$	$32{,}71^{bc}$	$1{,}18^{c}$	$6{,}00^{b}$	$27{,}38^{f}$	$17{,}22^{de}$	$19{,}23^{d}$	$5{,}5^{e}$	$21{,}35^{cd}$
NAA	1000	$14{,}6^{ab}$	$60{,}93^{bc}$	$41{,}40^{c}$	$0{,}79^{a}$	$6{,}00^{b}$	$16{,}81^{bcd}$	$13{,}39^{bc}$	$14{,}02^{bc}$	$3{,}0^{bc}$	$15{,}92^{abcd}$
NAA	5000	$8{,}0^{ab}$	$62{,}52^{ab}$	$51{,}36^{cd}$	$0{,}85^{a}$	$3{,}44^{a}$	$15{,}43^{bc}$	$10{,}83^{a}$	$10{,}66^{a}$	$3{,}0^{cd}$	$22{,}49^{d}$
GA3	1000	$42{,}6^{b}$	$76{,}42^{c}$	$56{,}47^{d}$	$0{,}83^{a}$	$3{,}75^{a}$	$14{,}49^{ab}$	$10{,}86^{ab}$	$10{,}30^{ab}$	$2{,}0^{a}$	$18{,}26^{bcd}$
GA3	5000	$14{,}6^{ab}$	$96{,}75^{d}$	$81{,}72^{e}$	$0{,}89^{a}$	$5{,}50^{b}$	$23{,}10^{ef}$	$14{,}64^{cd}$	$12{,}65^{abc}$	$4{,}0^{d}$	$13{,}81^{abc}$
Control	0	$41{,}2^{b}$	$55{,}57^{ab}$	$30{,}12^{ab}$	$0{,}93^{b}$	$6{,}00^{b}$	$18{,}84^{cde}$	$13{,}18^{bc}$	$13{,}31^{abc}$	$2{,}67^{ab}$	$10{,}32^{a}$
F values		1,993	12,09	25,617	17,471	9,316	11,898	9,865	8,77	35,778	23,273
Significant		0,044	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000

applied seedlings is 87% greater than that of the seedlings in the control group, in terms of stem length without branches, and 38% greater in total length. However, the seedlings produced as a result of other applications are either shorter than the control group or included in the same homogenous groups with the control group according to the result of the Duncan test.

The highest values of seedling diameter and number of leaves were acquired with the 5000 mg/L IBA application; however, one of the greatest values of the number of leaves was obtained in the control group. As a result, it can be concluded that the hormone applications do not have a positive effect, especially on the number of leaves.

When the effect of hormone applications on the leaf size is analyzed, it is observed that the IBA applications have a high effect on the leaf size and width. It was observed that the leaves exposed to the 1000 mg/L IBA application are 55% longer and 45% wider compared to the control group. The leaves exposed to the 5000 mg/L IBA application are 31% longer and 44% wider than the control group. It is observed that the 5000 mg/L IBA application is highly effective in terms of total leaf size. The leaves produced in the control group are 18.84 mm, while this figure is 27.38 mm in the 5000 mg/L IBA application and 23.1 mm in the 5000 mg/L GA3 application, which is highly effective in terms of increase in length of the seedlings.

Even though the stem length is an important indicator of the sapling quality, root/stem ratio is very important for a healthy sapling. The saplings that can generate hairy roots are generally accepted to be healthier, and the saplings that can generate taproot in a short span of time reach ground water more easily in the natural environment, and thus, their chance of survival increases.

Accordingly, root generation is one of the most important sapling quality indicators. According to the results of the study, the saplings in the control group developed 2.67 roots with an average length of 10.32, while the saplings receiving the 1000 mg/L IBA application developed 4 roots with an average length of 54.02. The saplings that received the 5000 mg/L IBA application had 5.5 roots with an average length of 21.35. The saplings that received the 5000 mg/L GA3 application developed 4 roots with an average length of 13.81.

The results of the study show that the hormone applications have a great effect on root development. The fact that only the number of roots of the seedlings developed with the 1000 mg/L GA3 application is lower than that of the control group, while all of the applications developed longer roots compared to the control group, and the roots of the saplings developed with the 1000 mg/L IBA application are more than five times longer than those of the control group indicates that the hormone applications have a great effect on root development.

4. Discussion

The results of the study demonstrate that cuttings with at least one bud must be used in order to produce *M. officinalis* using stem cuttings. No rooting developed in the stem cuttings without buds.

The results of the applications show that the auxin group of hormones (IAA, IBA, and NAA), the subject of this study, do not have an apparent effect on rooting rate but do have an effect on the morphological characteristics of newly generated plants. Root development in particular reached significantly different values in the plants that received the auxin group of hormones.

The process of adventitious root formation is influenced by a number of internal and external factors. Among the internal factors, the most important role is ascribed to phytohormones, especially the auxins. It is generally accepted that auxins have a certain role in the rooting initiation [15]. Auxins control growth and development in plants, including lateral root initiation and root gravity response. Many studies have shown that exogenous application of auxins results in increased initiation of lateral roots and that lateral root development is highly dependent on auxin and auxin transport [16].

The effects of auxin group of hormones on rooting and plant development have been discussed in several studies. Alvarez et al. [17] analyzed the effectiveness of IAA and IBA in *Malus pumila*; Štefančič et al. [15] studied the effectiveness of IAA and IBA in *Prunus* spp. as well as IBA and NAA in *Pseudotsuga menziesii*; Hossain et al. [18] analyzed the effectiveness of IBA in *Swietenia macrophylla* and *Chukrasia velutina*;

Hussain and Khan [19] analyzed the effectiveness of IAA and IBA in *Rosa* species; Ozel et al. [20] analyzed the effectiveness of IAA and NAA in *Centaurea tchihatcheffii*; Chhun et al. [16] researched the effectiveness of IAA, IBA, and NAA in *Oryza sativa*; De Klerk et al. [21] analyzed the effectiveness of IAA, IBA, and NAA in *Malus*; Martin [22] studied the effectiveness of IBA in *Holostemma ada-kodien*; Nordström et al. [23] researched the effectiveness of IAA and IBA in *Pisum sativum*; Tchoundjeu et al. [24] analyzed the effectiveness of IBA in *Prunus Africana*; Swamy et al. [25] studied the effectiveness of IBA and NAA in both *Robinia pseudoacacia* and *Grewia optiva*. The studies show that, in general, the auxin group of hormones has an effect on rooting. This result is in conformity with the results of this study.

Gibberellins are in the third place with a 17% share among the most commonly used herbal hormones within the natural plant growth regulators. Commercially the most common gibberellin is GA3, and it is used to increase the length of the plant or to enhance plant yield [26]. The results of the study reveal that the length of seedlings receiving the 5000 mg/L GA3 application is 2.71 times greater than that of the control group in terms of stem length without branches and 74% greater in total length. This result is also in compliance with the literature results.

Effect of GA3 on rooting was also analyzed in several studies. The efficiency of GA3 on *Prunus avium* L. and *Prunus mahaleb* was analyzed by Hepaksoy [27], by Aygün and Dumanoğlu [28] on *Cydonia oblonga*, by Coşge et al. [29] on *Capparis ovata* and *Capparis spinosa*, and by Selby et al. [30] on *Picea sitchensis*. However, no apparent GA3 efficiency on rooting was detected in several species.

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Research Article

High Efficiency Secondary Somatic Embryogenesis in *Hovenia dulcis* Thunb. through Solid and Liquid Cultures

Jingli Yang,[1] Songquan Wu,[2] and Chenghao Li[1]

[1] *State Key Laboratory of Forest Genetics and Tree Breeding (Northeast Forestry University), 26 Hexing Road, Harbin 150040, China*
[2] *Key Laboratory of Nature Resource of Changbai Mountain and Functional Molecular, Ministry of Education, Yanbian University, Park Road 977, Yanji, Jilin 133002, China*

Correspondence should be addressed to Chenghao Li; chli0@daum.net

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Embryogenic callus was obtained from mature seed explants on medium supplemented with 2,4-dichlorophenoxyacetic acid. Primary somatic embryos (SEs) can only develop into abnormal plants. Well-developed SEs could be obtained through secondary somatic embryogenesis both in solid and liquid cultures. Temperature strongly affected induction frequency of secondary embryogenesis. Relatively high temperature (30°C) and germinated SEs explants were effective for induction of secondary somatic embryos, and low temperature (20°C) was more suitable for further embryo development, plantlet conversion, and transplant survival. Somatic embryos formed on agar medium had larger cotyledons than those of embryos formed in liquid medium. Supplementing 0.1 mg L^{-1} 6-benzyladenine (BA) was effective for plant conversion; the rate of plant conversion was 43.3% in somatic embryos from solid culture and 36.5% in embryos from liquid culture. *In vitro* plants were successfully acclimatized in the greenhouse. The protocol established in this study will be helpful for large-scale vegetative propagation of this medicinal tree.

1. Introduction

Somatic embryogenesis is the process by which somatic cells develop into plants through characteristic morphological embryo stages. Somatic embryogenesis has many potential advantages for mass propagation and genetic improvement of hardwood trees. Clonal propagation through somatic embryogenesis can shorten the time needed for breeding and can improve the uniformity and quality of nursery stock [1]. The induction of somatic embryos (SEs) requires only a single hormonal signal to induce a bipolar structure capable of forming a complete plant [2]. Secondary somatic embryogenesis is a phenomenon whereby new SEs are initiated from SEs. For some species, secondary somatic embryogenesis offers the advantages of a high multiplication rate, independence from explants source effects, and repeatability. Well-developed SEs can be obtained through secondary somatic embryogenesis on medium without plant growth regulators, as described for Rosa hybrid [3], *Chrysanthemum* [4], and *Piper nigrum* [5]. The induction of secondary SEs critically affects the rate of plant conversion. Furthermore, the embryogenesis can be maintained for a prolonged period by repeated cycles of secondary embryogenesis [6].

Hovenia dulcis Thunb. (Semen Hoveniae), a member of Rhamnaceae, is a woody species mainly found in China, Korea, Japan, and India [7]. The fruit, seeds, leaves, roots, and bark of *H. dulcis* are all used in traditional Chinese medicine. It is well known for treating liver diseases [8], and several studies have demonstrated that extracts of *H. dulcis* or its purified compounds can serve as detoxifying agents for alcohol poisoning [8, 9]. In addition, the wood grain is beautiful, and the wood from these hardwood trees is suitable for furniture. Traditionally, *H. dulcis* is propagated by seed or via root and wood cuttings. These methods are inefficient because seeds have impermeable seed coats that severely inhibit germination and propagation by wood and root cuttings are rather difficult to form adventitious buds or roots regeneration from cuttings.

Development of an advanced propagation technology such as somatic embryogenesis might be an efficient method for biotechnological improvement of *H. dulcis*. *In vitro*

Table 1: Effects of temperature and developmental stage of SEs on induction of secondary SEs in *H. dulcis* after 6-week culture on MS agar medium without plant growth regulators[X].

Temperature (°C)	Secondary SE induction frequency (%)		
	Heart-shaped SEs	Cotyledonary SEs	Germinated SEs
20	0[g]	3.3[f]	13.3[e]
25	0[g]	30.0[d]	46.7[c]
30	10.0[e]	66.7[b]	86.7[a]

[X]Values with different letters are significantly different according to Duncan's multiple range test at the 5% level.

Table 2: Effect of temperature on production of secondary SEs from germinated SEs of *H. dulcis* after 6-week culture on MS agar medium without plant growth regulators[X].

Temperature (°C)	Number of secondary SEs/explant	Development stage of secondary SEs
20	5.7[c]	Cotyledonary
25	65.3[b]	Torpedo shaped
30	97.2[a]	Heart shaped

[X]Values with different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.

propagation of *H. dulcis* by organogenesis has been reported [10]. Our present study describes methods to obtain normal SEs and plantlet conversion following secondary somatic embryogenesis via culture of *H. dulcis* in agar and liquid media.

2. Materials and Methods

2.1. Somatic Embryo Induction. Seeds were collected from the mature fruit of wild *H. dulcis* in the Yangyang County of Kangwon in the republic of Korea. The seeds were first soaked in concentrated sulfuric acid for 20 min and then rinsed for 30 min with running water. The seed coats were then removed and the mature seeds soaked in 70% (v/v) ethanol for 1 min, sterilized in 2% (v/v) sodium hypochlorite solution for 20 min, and then rinsed four times with sterile distilled water.

The sterilized seeds were cultured on Murashige and Skoog (MS) [11] agar medium with 3.0% (w/v) sucrose supplemented with 1.0 mg L^{-1} 2,4-D (Sigma-Aldrich, USA). After 8 weeks, induced friable, fast-growing calli were selected and transferred to plant growth regulator-free MS agar medium containing 3% (w/v) sucrose for somatic embryo induction. During primary somatic embryogenesis, embryogenic calli frequently formed from the germinated SEs on PGR-free medium. The cultures were performed in a 250 mL Erlenmeyer flask containing 50 mL medium. All media were adjusted to pH 5.8 before adding 8 g L^{-1} plant agar (Duchefa, Haarlem, the Netherlands) and sterilized by autoclaving at 1.1 kg cm^{-2} (121°C) for 20 min. The culture room was maintained at 25 ± 1°C with a 16 h photoperiod at 36 μmol·m^{-2}·s^{-1} (cool white fluorescent tubes).

2.2. Secondary Somatic Embryogenesis from Solid and Suspension Culture. For induction of secondary somatic embryogenesis, heart-shaped, cotyledonary, and germinated primary SEs were transferred to MS agar medium containing 3% (w/v) sucrose and cultured at 20°C, 25°C, or 30°C, respectively. Eight germinated SEs were cultured per petri dish. Each experimental unit consisted of five dishes with three replicates. After 6 weeks, the percentage of secondary SE induction, number of secondary SEs per explants, and development stage of SEs were recorded. For further development, the secondary SEs were transferred to new MS agar medium containing 3.0% (w/v) sucrose and cultured at 20°C.

For secondary SE induction in suspension culture, germinated SEs were transferred to 50 mL MS liquid medium and cultured at 20°C, 25°C, or 30°C, respectively. Liquid medium was subcultured at 2-week intervals. Twenty germinated SEs were placed in each flask with 50 mL MS liquid medium by 20 replications. After 6 weeks, the induction frequency [Number of flasks formed secondary SEs/Number of flasks cultured × 100%] and fresh weight of embryogenic cell clumps in a flask were recorded. To induce secondary SE formation, embryogenic cell clumps were filtered through a 200 μm stainless steel screen to remove larger clumps, and about 200 mg of cell clumps was inoculated in a flask and cultured at 20°C. After 6 weeks, the developmental stage and number of secondary SEs in a flask were recorded. To obtain the fresh weights of cell clumps and number of SEs, a 1.0 mL aliquot of the embryo suspension from each treatment was removed using a modified pipette tip (i.e., 5 mm was cut from the end of a standard 1 mL pipette tip to widen the aperture to prevent clogging), and fresh weight and number of SEs were measured. The overall number and growth of SEs were then calculated by multiplying the measured data by the total volume of the vessel medium. All cultures were agitated at 100 rpm on a gyrating shaker.

2.3. Plant Conversion. Germinated secondary SEs were selected and transferred to 1/3-strength MS agar medium containing 1.0% (w/v) sucrose and supplemented with 0.1 to 2.0 mg L^{-1} BA and then cultured at 20°C or 25°C. After 2 months, the percentages of plantlet conversion were

Table 3: Effect of temperature on induction of embryogenic cells from germinated SEs of *H. dulcis* after 6-week culture on MS liquid medium without plant growth regulators[X].

Temperature (°C)	Embryogenic cell induction frequency (%)[Y]	Fresh weight of embryogenic cell clumps (mg/flask)
20	0[c]	—
25	65[b]	97.3[b]
30	100[a]	894.6[a]

[X]Values with different letters in a column are significantly different according to Duncan's multiple range test at the 5% level.
[Y]Embryogenic cell induction frequency was calculated by Number of flasks formed secondary.
SEs/number of flasks cultured × 100%.

recorded. Ten germinated SEs were cultured in a vessel. Each experimental unit consisted of five flasks with three replicates.

2.4. Transplantation. Regenerated plantlets with more than five leaves were selected and transferred to pots containing autoclaved soil mixture (1 : 3, sand : soil) in a growth chamber. Pots were covered with polythene bags to maintain high humidity and incubated in a growth chamber at 20°C or 25°C. The bags were perforated, and covers were removed after 3 weeks when the plants showed new leaves. After 2 months, the survival rate of plants was measured. Twenty plants were planted in the soil mixture (1 : 3, sand : soil), and each experiment was repeated three times.

2.5. Statistical Analysis. The data variance (ANOVA) was analyzed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Means differing significantly were compared using Duncan's multiple range test at a 5% probability level.

3. Results and Discussion

3.1. Somatic Embryo Induction. Callus was induced from seed explants after 2-week culture at 25°C and continued to grow rapidly. After 2 months in culture, yellowish-white embryogenic calli formed on the surface of the calli. The embryogenic callus induction frequency reached presumably 10% after 3 months of culture. When transferred to plant growth regulator-free agar medium, embryogenic calli differentiated into SEs, most of which could germinate but developed abnormally. These abnormal SEs rarely converted into plantlets. The appearance of abnormal embryos and subsequent low frequency of plantlet conversion are severe constraints preventing practical application of this technology [1].

3.2. Secondary Somatic Embryogenesis on Solid Culture. Secondary SEs mainly formed from hypocotyls of germinated primary SEs after 2 weeks at 25°C (Figures 1(a) and 1(b)). The development of most of these SEs followed the normal stages of zygotic embryos from globular, heart-shaped to cotyledonary SEs and germinated (Figure 1(c)). A new cycle of secondary somatic embryogenesis initiated from primary SEs. Through this cyclic SE induction process, stable embryogenic cultures were maintained for more than 1 year. Similar observations have been reported for other species such as *Schisandra chinensis* [12, 13]. Pinto et al. [14] reported that repetitive secondary SE induction was efficient in *Eucalyptus globulus*, which produces a larger number of SEs than from primary SEs, thus increasing the potential rate of plantlet conversion.

The temperature and developmental stage of SEs significantly affected the induction of secondary SEs. Relatively high temperature (30°C) and germinated SEs were effective for induction of secondary SEs (Table 1). At higher temperatures, more secondary SEs were induced, accomplished with delayed SE development (Table 2). Kamada et al. [15] showed similar results for cultures of early-stage somatic embryos in carrots. In general, high temperature stress can turn somatic cells into embryogenic cells [15, 16].

3.3. Secondary Somatic Embryogenesis in Suspension Culture. To induce secondary embryogenesis from liquid cultures, germinated SEs were transferred to MS liquid medium and cultured at 20°C, 25°C, or 30°C. Numerous embryogenic cell clumps were formed from SEs after 4 weeks of culture. Similar to semisolid culture, the frequency of embryogenic cell clump formation was highest at 30°C (Table 3). These embryogenic cell clumps propagated quickly at 30°C, and their multiplication efficiency was presumably 9-fold higher than culture at 25°C (Table 3). However, no SEs developed at 25°C and 30°C. When cultured at 20°C, however, embryogenic cells developed into SEs and germinated after 4 weeks of culture (Figure 2(a)).

For synchronous development of SEs, cell suspensions were filtered through a 200 μm sieve to remove larger clumps, and ~200 mg of filtered cell clumps was transferred to each flask and cultured at 20°C (Figure 2(b)). After 6 weeks, approximately 2363 SEs formed from 200 mg of cell clumps in a flask (Figure 2(c)). Our results showed that the frequency of SE formation in liquid culture was much higher than that in solid culture. The structure of cotyledonary SEs that developed in suspension culture differed somewhat from those formed in solid culture. SEs formed on solid culture had larger cotyledons and a more intact epidermis compared to embryos formed in liquid culture (Figures 2(d) and 2(e)).

3.4. Plantlet Conversion. Germinated SEs were transferred to 1/3-strength MS medium containing 1% (w/v) sucrose for conversion to plantlets. After 2 months of culture, the plantlet conversion frequency was examined. Most SEs could not convert into plantlets at 25°C from both agar and liquid

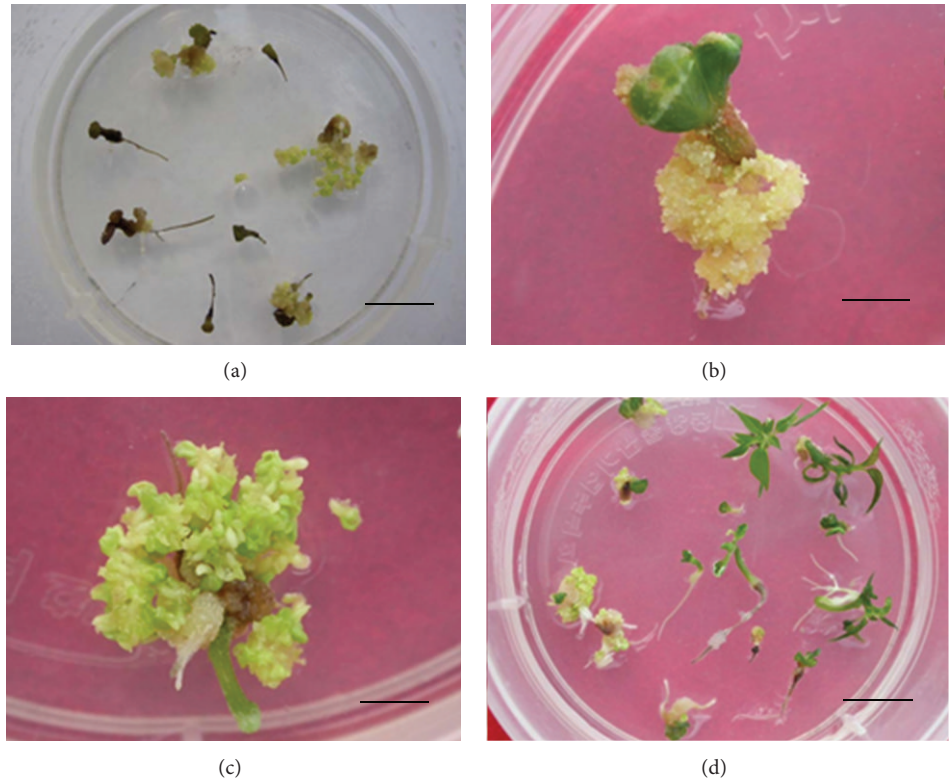

Figure 1: Cyclic somatic embryogenesis from germinated SEs on agar medium. (a) Embryogenic calli formed directly from germinated SEs on medium without plant growth regulators. (b) Close view of embryogenic calli formed from the primary SEs. (c) Embryogenic calli developed into cotyledonary SEs. (d) Plantlets from germinated SEs. Bars = 15 mm (a), 2 mm (b), 2 mm (c), and 15 mm (d).

Table 4: Effects of BA supplementation on plantlet conversion of *H. dulcis* secondary SEs after 2 month-culture on 1/3-strength MS agar medium at 20°C[X].

BA concentration (mg l^{-1})	Plantlet conversion frequency of secondary SEs (%)	
	SEs cultured on agar medium	SEs cultured in liquid medium
0	36.1[b]	17.0[d]
0.1	43.3[a]	36.5[b]
0.5	28.0[c]	13.3[de]
1.0	6.7[e]	0[f]
2.0	3.3[e]	0[f]

[X]Values with different letters are significantly different according to Duncan's multiple range test at the 5% level.

media cultured germinated SEs (data not shown), whereas relatively low temperature (20°C) was suitable for plantlet conversion (Figures 1(d) and 2(f)). Indeed, such a low temperature is required to increase the rate of plantlet conversion [17], possibly because of a decreased content of inhibitory substances such as abscisic acid and increased levels of gibberellic acid to promote germination. Our result showed SEs culture medium significantly affected plantlet conversion ($P < 0.001$), which SEs derived from agar medium showed higher conversion frequency than that of SEs from liquid culture at the same culture conditions (Table 4). Similar results were also reported in Siberian ginseng, in which the rate of plant conversion was 97% in somatic embryos from callus culture and 76% in embryos from liquid culture [18]. This difference might be a result of the different culture conditions used during SE development.

The concentration of BA also affected plantlet conversion ($P < 0.001$), and 0.1 mg L^{-1} BA was effective for plantlet conversion to secondary SEs in both agar and liquid media (Table 4). A total of 43.3% of SEs formed from solid culture was converted to plantlets, but only 36.5% of SEs from suspension culture converted into plantlets on medium containing 0.1 mg L^{-1} BA (Table 4).

3.5. Transplantation. When *in vitro* plantlets were cultured in the greenhouse for 4 days, new roots appeared from the plantlets cultured at 20°C. After ~10 days, new leaves

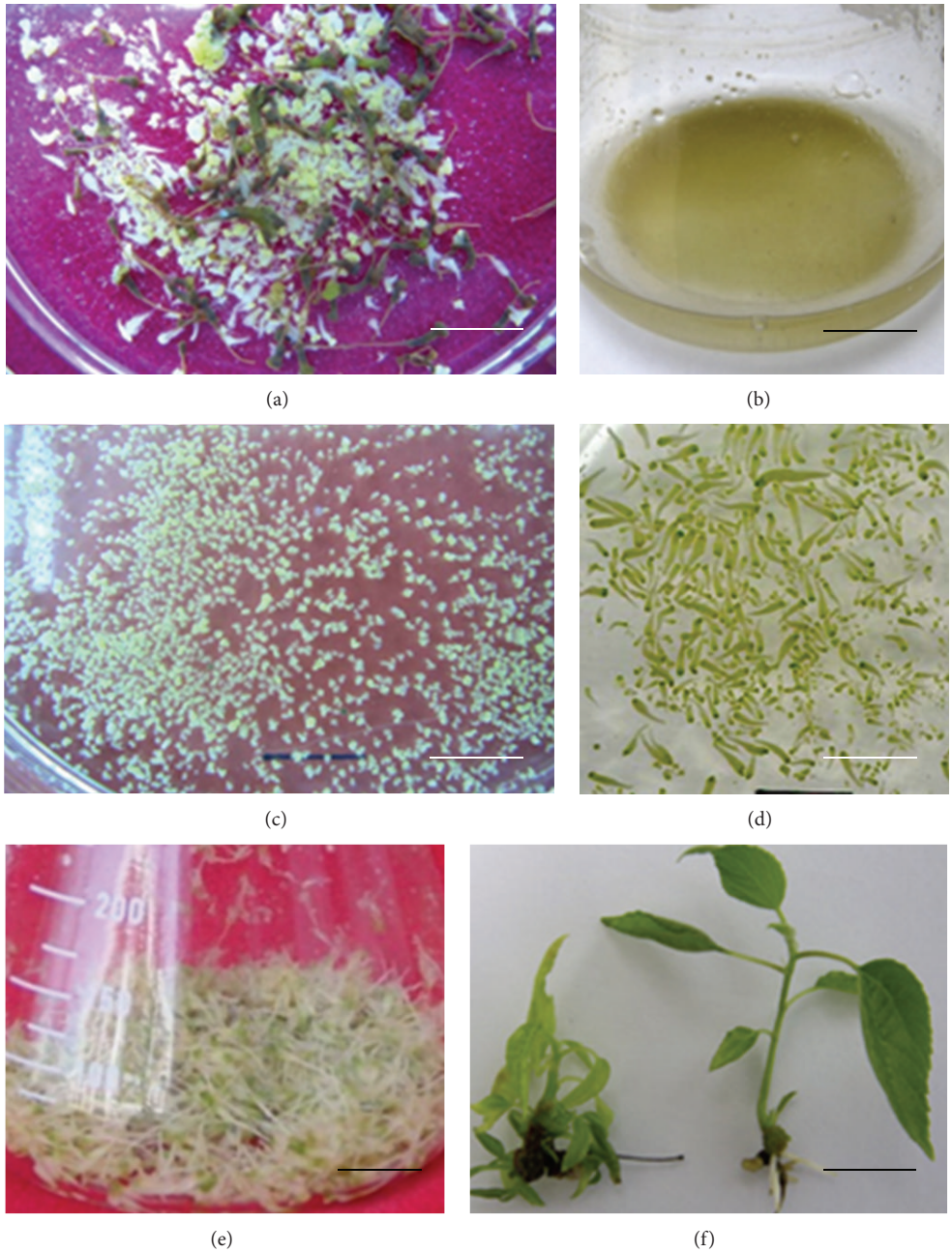

Figure 2: Cyclic somatic embryogenesis from germinated SEs on liquid medium. (a) Secondary SEs (bright white) induced directly from primary germinated SEs (black) in liquid medium 4 weeks after being cultured in MS liquid medium without PGRs. (b) Collected embryogenic cell clumps induced from germinated SEs in liquid medium. (c) Numerous heart-shaped SEs which developed after embryogenic calluses were cultured in MS liquid medium without PGRs for 6 weeks. (d) Cotyledonary SEs after embryogenic calluses were cultured in liquid medium for 8 weeks. (e) Germinated SEs after embryogenic calluses were cultured in liquid medium for 10 weeks. (f) Plantlets from germinated SEs on agar medium. Bars = 10 mm (a), 15 mm (b), 10 mm (c), 10 mm (d), 15 mm (e), and 10 mm (f).

began growing. The survival rate reached 82% after 2 months (Figure 3).

4. Conclusion

There are few reports on the effect of culture temperature on secondary SE formation and plant regeneration. The most significant result of our investigation is that a relatively high temperature (30°C) is better for induction of secondary SEs, whereas a relatively low temperature (20°C) is suitable for plantlet conversion. In conclusion, we report the optimal temperature conditions necessary for secondary somatic embryogenesis and plantlet conversion in *H. dulcis*. The protocol established in this study will be helpful for the

Figure 3: *In vitro* plantlets 2 months after acclimatization in soil. Bars = 30 mm.

conservation and large-scale vegetative propagation of *H. dulcis*.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid
BA: 6-benzyladenine
MS: Murashige and Skoog
SE: Somatic embryo
PGR: Plant growth regulator.

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Research Article

Comparison of Different Methods for Separation of Haploid Embryo Induced through Irradiated Pollen and Their Economic Analysis in Melon (*Cucumis melo* var. *inodorus*)

Gökhan Baktemur,[1] Hatıra Taşkın,[2] and Saadet Büyükalaca[3]

[1] *Department of Biology, Faculty of Arts and Sciences, University of Osmaniye Korkut Ata, 80000 Osmaniye, Turkey*
[2] *Department of Plant Production and Technologies, Faculty of Agricultural Sciences and Technologies, University of Niğde, 51240 Niğde, Turkey*
[3] *Department of Horticulture, Faculty of Agriculture, University of Çukurova, 01330 Adana, Turkey*

Correspondence should be addressed to Hatıra Taşkın; hatirataskin1@gmail.com

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Irradiated pollen technique is the most successful haploidization technique within *Cucurbitaceae*. After harvesting of fruits pollinated with irradiated pollen, classical method called as "inspecting the seeds one by one" is used to find haploid embryos in the seeds. In this study, different methods were used to extract the embryos more easily, quickly, economically, and effectively. "Inspecting the seeds one by one" was used as control treatment. Other four methods tested were "sowing seeds direct nutrient media," "inspecting seeds in the light source," "floating seeds on liquid media," and "floating seeds on liquid media after surface sterilization." Y2 and Y3 melon genotypes selected from the third backcross population of Yuva were used as plant material. Results of this study show that there is no statistically significant difference among methods "inspecting the seeds one by one," "sowing seeds direct CP nutrient media," and "inspecting seeds in the light source," although the average number of embryos per fruit is slightly different. No embryo production was obtained from liquid culture because of infection. When considered together with labor costs and time required for embryo rescue, the best methods were "sowing seeds directly in the CP nutrient media" and "inspecting seeds in the light source."

1. Introduction

Melon (*Cucumis melo* L.), which belongs to the *Cucurbitaceae* family, is one of the important vegetables because of a rapid increase in its production and nutrient value. Melon is rich in terms of protein, minerals and vitamins, such as Vitamin A (500–4200 IU/100 g) and Vitamin K (130–330 mg/100 g) [1]. In recent years, it has become widespread to use melon in fruit salad and fruit juice as well as consuming it freshly. Moreover, it is used in other branches of the food industry (pastry, jam, ice-cream, and fruit yogurt). Its immature fruits can be used in making pickles and can be used in making soup in the Far East. Some melon species are also known to be used as ornamental plants. It is also common in perfumery and cosmetic industry such as in shampoos and perfumes [2]. Melon holds a great economic value in the world in terms of human nutrition and 28 million ton production in 1.3 million ha area annually. Turkey ranks second among the world's melon-producing countries with 103000 ha area and total production of 1.75 mt [3].

Obtaining homozygote pure lines through conventional breeding techniques in melon requires a long period such as 10–12 years, and 100% homozygous cannot provide because of open pollinations. Therefore, these techniques can conduct with more quick and effective *in vitro* techniques such as "haploidy techniques." It is possible to get 100% homozygote pure lines in one year by obtaining plants that have haploid chromosome number and doubling their chromosome numbers with colchicine. Hence, homozygotation process can be shortened for 1 to 2 years. Toward 1990s, *in sitü* haploid

embryo stimulation by pollination with gamma irradiated pollens and germination of these embryos in special nutrient media were studied, and this process was performed in cucumber [4, 5], melon [6–8], watermelon [9, 10], and squash [11]. Because the number of the plants obtained was adequate and regular in melon and cucumber, this method was started to be used in breeding program of melon and cucumber. The most common method to obtain haploid embryo is "inspecting the seeds one by one." However, this method takes long time and needs laboratory staff. There are a limited number of studies on the use of different methods. In a study conducted for obtaining double haploid lines through irradiated pollen technique in cucumber, embryos in seeds were determined through X-ray [12, 13]. However, this method is not widely used because it requires special equipment as an X-ray source. Lotfi et al. [14] planted the seeds into the fluid culture, and the seeds germinated. In some of our previous studies, the seeds were examined in a light source; the ones which were detected to have embryo were separated and opened.

The main objective of this study is to explore the time and labor cost of the four methods that are "sowing seeds into direct nutrient media," "inspecting seeds in the light source," "floating seeds on liquid media," and "floating seeds on liquid media after surface sterilization" as an alternative to the method of "inspecting the seeds one by one," to compare the time of the best practice and unit costs counted for each method, and to put forward the least-cost method.

2. Material and Methods

2.1. Material. Y2 and Y3, the third backcross (BC3) population of Yuva (local recurrent cultivar) selected from a melon breeding project conducted at the Department of Horticulture, Faculty of Agriculture, University of Çukurova, were used as plant materials in this study. Resistance melon genotypes to 0, 1, and 3 races of Fusarium wilt and Yuva melon genotypes were crossed to obtain BC3 population.

2.2. Methods

2.2.1. Greenhouse Practices and Embryo Stimulation. This study was conducted in a 326 m^2-plastic greenhouse belonging to the Department of Horticulture, Faculty of Agriculture, University of Çukurova. Seeds were sown plugs containing soil mixture (2 volumes peat : 1 volume perlite) in February. Seedlings were transferred to plastic greenhouse with double-row systems (50 × 50 × 100 cm space) in March. Throughout the growing period, normal horticultural cultivation practices were implemented. Pollination with irradiated pollen was performed between 16th April and 16th May, which was suitable for the spring breeding period. Male flowers were collected one day before anthesis (the stage when petals started to change their color from green to yellow). After they were separated from petals and partially sepals, they were put into glass petri dishes for irradiation. Female flowers were emasculated with pens and closed with cellophane bags to prevent open pollination on the same day. Irradiation was performed at the Department of Radiation Oncology, Faculty of Medicine, University of Çukurova, with 300 Gy gamma ray coming from Co^{60} [7]. Irradiated male flowers were waited in room temperature during the night. The next day, female flower was emasculated the day before being pollinated by irradiated male flowers. After pollination, flowers were closed with cellophane bags again to pollen contamination. In the forthcoming days, the state of pollination was checked, and cellophane bags were removed in the period when the ovary of the female flower began to swell and the stigma became dry (Figure 1).

2.2.2. Embryo Extraction. Melon fruits were harvested between the dates of 11th May and 21st June, 21–25 days after pollination. After harvested fruits were washed with tap water and dried, they were placed in a sterile jar and disinfected with 96% ethyl alcohol through dry burning method (Figure 1). Then, the fruits were separated into two by lengthwise cutting, and the seeds inside were extracted and put into sterile petri dishes. Five different methods were tested for the embryo rescue (Figure 2).

Method 1. All the seeds were opened one by one under stereo binocular microscope and checked whether they contained embryo. Finding embryos were placed in glass culture tubes containing E20A nutrient medium [6, 12] with 8 g L^{-1} agar.

Method 2. Seeds without exposure to any treatment were directly sown into sterile plastic 5-cm-diameter petri dishes containing CP medium [15] with 30 g L^{-1} sucrose, 8 g L^{-1} agar, 0.08 mg L^{-1} Vitamin B12, 0.02 mg L^{-1} IAA and E20A medium with 8 g L^{-1} agar. The sown seeds were observed regularly for the first 20 days. Germinated embryos were transferred from petri dishes to glass culture tubes.

Method 3. Seeds extracted from fruits in laminar flow hoods were placed in sterile empty plastic petri dishes and carefully examined on a mechanism prepared with fluorescent light, and seeds containing embryo were selected and transferred to another sterile empty plastic petri dishes. Embryos were excised from these seeds under stereo binocular microscope, and the seeds were placed into culture tubes containing nutrient medium, as in Method 1.

Method 4. Seeds, without exposure to any treatment, were directly sown in sterile plastic petri dishes containing liquid nutrient media (80–120 seeds/petri). As a nutrient medium, again, CP medium with 30 g L^{-1} sucrose, 0.08 mg L^{-1} Vitamin B12, 0.02 mg L^{-1} IAA and E20A medium without agar were used. Sown seeds were observed for 20 days, and when haploid embryos were seen, they were transferred to culture tubes containing nutrient media.

Method 5. All the seeds were waited in 15% sodium hypochlorite for 10 minutes for surface sterilization and washed 3–5 times with sterile water. Then, they were sown into glass petri dishes containing CP medium with 30 g L^{-1} sucrose, 8 g L^{-1} agar, 0.08 mg L^{-1} Vitamin B12, 0.02 mg L^{-1} IAA and E20A

Figure 1: (A) Pollination of female flowers with irradiated male flowers, (B) closing of pollinated female flowers with cellophane bags to prevent pollen contamination, (C) harvested melon fruits, (D) disinfection of melon fruits with 96% ethyl alcohol through dry burning method.

medium with 8 g L^{-1} agar. Seeds, observed for 10 days, were transferred to nutrient media in glass culture tubes after the haploid embryo detection.

All cultures were incubated in the growing room at 25°C temperature and under 8-hour dark and 16-hour light photoperiod conditions. The experiment was designed in a completely randomized experimental design with three replications and five fruits included per replication. The number of seeds in each fruit and those containing the embryo was detected, the required time for each method was recorded, and unit costs of each method were compared. Total embryo number per fruit, the number of embryos transforming into plant, the number of developed plants, amount of infection, time for opening a fruit, and cost of labor force per embryo were observed to compare the methods. Variance analysis was conducted to evaluate the results, and Tukey test was used for controlling the significance of the differences.

3. Results and Discussion

When the methods were compared, seed numbers per fruit were close to each other, and infection was not observed except in Method 4 during any stage of the study as shown in Table 1. Therefore, methods were compared in terms of embryo number per fruit. Method 1 was found to be most successful with 46 embryos $fruit^{-1}$, followed by Method 3 with 42 embryos $fruit^{-1}$ and Method 2 (CP) with 36 embryos $fruit^{-1}$. The number of obtained embryos was found to be 17 embryos $fruit^{-1}$ in Method 2 (E20A), 7 embryos $fruit^{-1}$ in Method 5 (CP), and 3 embryos $fruit^{-1}$ in Method 5 (E20A). Embryo germination could not be provided in Method 4 because of infection.

Average embryo number extracted from one fruit in different methods was given in Table 2. There was no statistical difference among Methods 1, 2 (CP), and 3. Accordingly, on average, 3.30 embryos $fruit^{-1}$ were obtained from Method 1, 3.10 embryos $fruit^{-1}$ from Method 3, and 2.40 embryos $fruit^{-1}$ from Method 2 (CP). The embryo number was 1.10 embryos per fruit in Method 2 (E20A); whereas the average embryo number was found to be 0.50 embryos per fruit in Method 5 (CP), it was 0.20 embryos per fruit in Method 5 (E20A).

To compare the methods in terms of time for obtaining the embryo, by keeping the time the fruit was cut, total time, seed extraction of each method, time for rescuing the embryo, and embryo number per fruit were identified (Table 3). In the present study, only seed extraction from the fruit was calculated as approximately 31.4 minutes for one fruit in each method. In the method of "inspecting seeds one by one" as the control treatment, roughly 3.3 embryos were obtained from one fruit, and the time for opening seeds in fruits, finding the embryos, and sowing on the media in culture tubes was about 103.5 minutes.

In Method 2, the rate of rescued embryos per fruit was 2.4, and all the seeds in each fruit were sown in sterile plastic petri dishes containing nutrient media in approximately 56.3 minutes. When compared with Method 1, there was no statistical difference in terms of the obtained embryos number, but this method has an advantage in terms of the time spent per fruit.

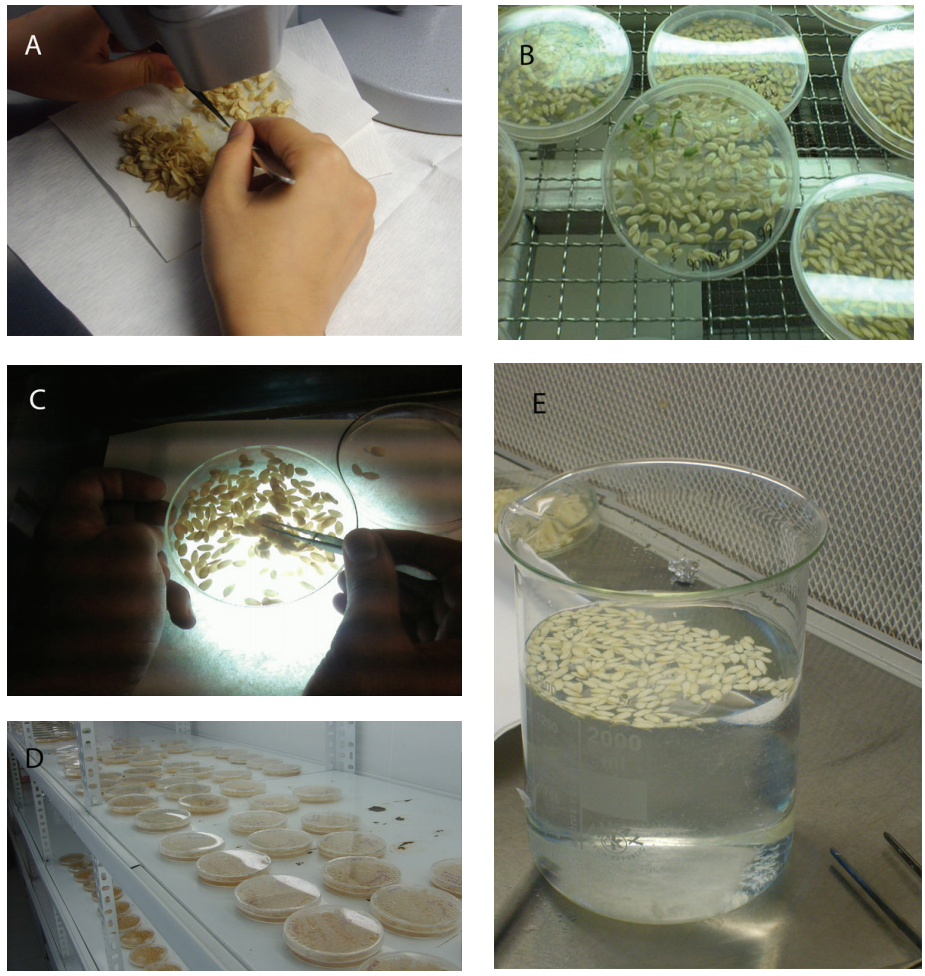

Figure 2: (A) Method 1. (B) Method 2. (C) Method 3. (D) Method 4. (E) Method 5.

Table 1: Comparison of seed number per fruit, embryo number, the number of embryos transforming into plant, the number of developed plants, and amount of infection in melon according to methods (Figure 3).

Methods	Seed number per fruit	Embryo number	GEN	GPN	IN
Method 1	1206	46	44	44	—
Method 2 (CP)	1132	36	36	36	—
Method 2 (E20A)	1293	17	17	17	—
Method 4 (CP)	—	—	—	—	—
Method 4 (E20A)	—	—	—	—	—
Method 5 (CP)	1353	7	7	7	—
Method 5 (E20A)	1252	3	3	3	—
Method 3	1249	42	42	42	1

GEN: germinated embryo number.
GPN: growing plant number.
IN: infection number.

Seeds containing embryo could be easily selected on the light source in Method 3. These seeds were opened embryos were taken and transferred to the nutrient media. Approximately 3.1 embryos were rescued per fruit, and this method took roughly 48.8 minutes. When compared with Method 1, the process was completed in less than half of the time; when evaluated in terms of embryo number per fruit, it was found to be statistically in the same group with Method 2. When compared with the study conducted by Lotfi and Salahi [16], who stated that they examined more than 400 seeds in one hour, it was seen that roughly 1500 seeds were inspected per hour in our study. The same researchers noted

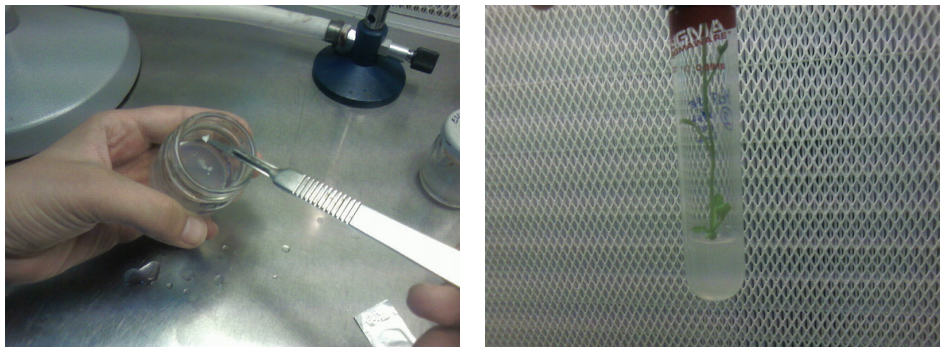

Figure 3: A haploid melon embryo and a plant obtained through germination of this embryo.

Table 2: Average embryo number per fruit obtained from different methods.

Methods	Embryos per fruit
Method 1	3.30 a
Method 2 (CP)	2.40 a
Method 2 (E20A)	1.10 b
Method 5 (CP)	0.50 b
Method 5 (E20A)	0.20 b
Method 3	3.10 a
LSD % 5	1.194

that opening fruits one by one took hours. In our study, we conducted with about similar seed number; opening one fruit was completed in 1.5–2 hours.

Another negative side of Method 1 is that embryos may become damaged in case it is performed by people with inadequate experience. Moreover, small and immature embryos at the globular stage may not be noticeable unless seen through a binocular microscope. Apart from these methods, in "after-sterilization sowing," all the seeds in each fruit were sown in petri dishes containing nutrient media in approximately 70.0 minutes. The most important reason of the increase in duration here is the time spent for sterilization of the seeds. In this method, embryo yield was quite low; the cause of which was thought that sterilization fluid passed through the unripe embryo shell and damaged the embryo.

Method 4 was unsuccessful because of infection. In a study carried out by Lotfi et al. [14], they remarked that infection occurred in some of the petri dishes, and they lost 30% of the fruits used because of this reason. These researchers examined two different sterilization methods in their study and stated that seed sterilization was more successful than fruit sterilization. In another study conducted by Lotfi and Salahi [16], they indicated that 22% of the seeds transferred to the fluid media got infected. They specified that losses due to infections were 7.8% in seeds disinfected by Clorox, whereas it was 35% in fruit disinfection. Infection is a big problem in fluid culture because infection can quickly spread from one seed to the media in a short time.

The cost of the 5 different methods used in obtaining embryos is rather important as well. For this reason, the number of haploid plants obtained through each method and total labor force cost for doing this were examined. With the calculations as basis, the cost of each obtained haploid plants was almost 30$, and 9.49 $/day was paid for the worker extracting seeds from the fruit, and 18.43 $/day was paid for the ones working in embryo rescue from the seeds. In cost account, daily working hour of a worker was calculated as 480 minutes. Because almost all obtained embryos transformed into plant in our study, embryo number was used also as plant number. Unit haploid plant cost was found by dividing calculated total cost by the obtained haploid plant number. By comparing the unit costs calculated for each method, the method with the least cost was determined (Table 4).

About 85.52 plants are produced, and $2565.6 is earned in Method 3. This method is followed by Method 2 (CP); the number of obtained plant was calculated as 46.26, and the cost was $1387.8. Similar number of plant was obtained as a result of Method 2 (E20A) whose embryo number was lower than Method 1. Furthermore, considering the labor cost, it was apparent that more income was gained. Thus, 21.20 plants and $636.00 were obtained in Method 2 (E20A). In Method 1, the number of the obtained plants was 21.96, and the income was $658.8. In Method 5 (CP), the number of plants was 5.33, and income was $159.9, whereas in Method 5 (E20A), the plant number was 2.13, and the income was $56.61. Considering that about 1000 people can work for a comprehensive improvement practice, when obtained data are calculated according to 1000 plants, Method 3 lasts shortest with 11.6 days; for this reason, the labor cost in this method will be very low, $213.78. Method 2 (CP) lasted 21.7 days, with a total cost of $399.93. Method 2 (E20A) lasted for 46 days with a cost of $847.78. Method 1 lasted for 46.1 days, with a cost of $849.62. Method 5 (CP) lasted 202.0 days and costed $3722.86. Method 5 (E20A) lasted 473.5 days, with a cost of $8726.60. As a consequence, when we examine the labor force cost necessary to obtain 1 haploid plant, it appears that $0.2 for Method 3, $0.4 for Method 2 (CP), $0.8 for Method 2 (E20A), $0.8 for Method 1, $3.7 for Method 5 (CP), and $8.7 for Method 5 (E20A) are required.

4. Conclusion

When embryo rescue methods were compared, the highest values in terms of embryo numbers per fruit were found in

Table 3: Average embryo number per fruit obtained through different methods and time.

Methods	Embryos/fruit	Time (seed extraction excluded-minutes/fruit)	Total time (minute/fruit)
Method 1	3.30 a	72.1	103.5
Method 2 (CP)	2.40 a	24.9	56.3
Method 2 (E20A)	1.10 b	24.9	56.3
Method 5 (CP)	0.50 b	45.0	70.00
Method 5 (E20A)	0.20 b	45.0	70.00
Method 3	3.10 a	17.4	48.80
Method 4 (CP)	—	5.1	36.5
Method 4 (E20A)	—	5.1	36.5

Table 4: Labor force and embryo costs according to time in different embryo rescue methods.

Methods	Embryo number/day	Income ($)	For 1000 plants		
			Time (day)	Labor ($)	Haploid plant/labor force ($)
Method 1	21.96	658.8	46.1	849.62	0.8
Method 2 (CP)	46.26	1387.8	21.7	399.93	0.4
Method 2 (E20A)	21.20	636.00	46.0	847.78	0.8
Method 3	85.52	2565.6	11.6	213.78	0.2
Method 5 (CP)	5.33	159.9	202.0	3722.86	3.7
Method 5 (E20A)	2.13	63.9	473.5	8726.60	8.7

Methods 1, 2, and 3, and there was no statistical difference among these methods. When the methods were compared in terms of duration, Method 1, which used as a control in the experiment, took the longest time, and Method 3 took the shortest time. This method lasted shorter than the other methods because the embryos of the seeds in petri dishes placed on a lighting mechanism seemed more easily. When evaluated in terms of cost, Method 3 provided the highest income, and Method 5 (E20A) brought the least income. As a consequence of the methods that were tried to extract haploid embryos more easily and effectively, many haploid embryos were obtained in a short time through Method 3 and Method 2 (CP). Improvement practices of local species in our country, which has a wide genetic potential in terms of melon, will gather pace, and the dihaploidization method will be used more commonly and effectively in our country's improvement practices.

Abbreviations

Y2 and Y3: Melon genotypes selected from the third backcross (BC3) population of Yuva (local recurrent cultivar)
CP: A nutrient medium developed by Chee et al. [15].

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Research Article

Comparative Studies on Cellular Behaviour of Carnation (*Dianthus caryophyllus* Linn. cv. Grenadin) Grown *In Vivo* and *In Vitro* for Early Detection of Somaclonal Variation

Jamilah Syafawati Yaacob, Rosna Mat Taha, and Arash Khorasani Esmaeili

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Correspondence should be addressed to Jamilah Syafawati Yaacob; jam_sya@yahoo.com

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The present study deals with the cytological investigations on the meristematic root cells of carnation (*Dianthus caryophyllus* Linn.) grown *in vivo* and *in vitro*. Cellular parameters including the mitotic index (MI), chromosome count, ploidy level (nuclear DNA content), mean cell and nuclear areas, and cell doubling time (Cdt) were determined from the 2 mm root tip segments of this species. The MI value decreased when cells were transferred from *in vivo* to *in vitro* conditions, perhaps due to early adaptations of the cells to the *in vitro* environment. The mean chromosome number was generally stable ($2n = 2x = 30$) throughout the 6-month culture period, indicating no occurrence of early somaclonal variation. Following the transfer to the *in vitro* environment, a significant increase was recorded for mean cell and nuclear areas, from $26.59 \pm 0.09\ \mu m^2$ to $35.66 \pm 0.10\ \mu m^2$ and $142.90 \pm 0.59\ \mu m^2$ to $165.05 \pm 0.58\ \mu m^2$, respectively. However, the mean cell and nuclear areas of *in vitro* grown *D. caryophyllus* were unstable and fluctuated throughout the tissue culture period, possibly due to organogenesis or rhizogenesis. Ploidy level analysis revealed that *D. caryophyllus* root cells contained high percentage of polyploid cells when grown *in vivo* and maintained high throughout the 6-month culture period.

1. Introduction

Carnation or *Dianthus caryophyllus* is a herbaceous perennial plant that can grow up to 80 cm tall, with grayish green or blue-green glaucous leaves. Carnation flowers are sweetly scented, about 3–5 cm in size (diameter), and are either produced singly or in a bunch. Carnation flowers are naturally bright pinkish-purple in colour, but other colourful cultivars of this plant had been developed such as carnations with white, red, green, and yellow flowers. The increasing demand for carnations has rendered this species to be a special candidate for mass propagation through tissue culture. Frey and Janick [1] reported on organogenesis observed from carnation petals cultured on MS [2] medium supplemented with 0.05 μM TDZ and 0.5 μM NAA. Ali et al. [3] showed *in vitro* shoot formation from apical and nodal meristems of carnation when cultured on an MS medium fortified with BAP. On the other hand, Watad et al. [4] observed *in vitro* shoot formation from internode explants cultured on an MS medium supplemented with TDZ and NAA. However, the effect of plant growth regulators, following the transfer of *D. caryophyllus* cells from *in vivo* to *in vitro* conditions and investigation at the cellular level, had not been reported before.

According to Karp [5], basic cytological technique is very important and useful for the determination of accurate chromosome number and structure and should be in routine use for regenerated plants in tissue culture. Cytological studies, that is, measurements of the mitotic index (MI), mean cell and nuclear areas, chromosome count, and cell doubling time (Cdt), were carried out to elucidate any differences or changes that occurred in *in vivo* and *in vitro* grown *D. caryophyllus* plants at the cellular level, enabling the easy and early detection of somaclonal variations. Cytology facilitates chromosomal and cell division studies in plants [6] as well as enables the detection of embryogenic callus from nonembryogenic callus [7] and *in vitro* flowering [8, 9]. Thus, the aims of the present study are to compare at the cellular level the characteristics of tissues obtained from *in vivo* and *in vitro* environments,

to detect any cellular changes when cells are transferred from the *in vivo* to the *in vitro* system, and to detect any occurrence of somaclonal variation at the cellular level.

2. Materials and Methods

2.1. Sterilization of Seeds and Determination of Standard Growth of Primary Roots. One hundred seeds of *Dianthus caryophyllus* Linn. cv. Grenadin bought from Yates Company, Australia, were surface-sterilized following standard tissue culture protocols [10] but with minor modifications. The seeds were washed using sterile distilled water, followed by treatments with 100%, 70%, and 30% (v/v) commercial bleach (Clorox) for 2 minutes at each concentration. Two drops of Tween-20 were also added during the treatment with 100% (v/v) Clorox to facilitate the sterilization process and reduce surface tension. The seeds were rinsed with sterile distilled water to remove excess Clorox, submerged in 70% (v/v) ethanol, and finally rinsed 3 times with sterile distilled water.

The seeds were then germinated on preautoclaved moist cotton wool and maintained in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark for 5 days. The growth of primary roots was monitored on a daily basis, whereby the length of the primary roots was measured at the same time every day to determine the standard growth of *Dianthus caryophyllus* primary roots. A graph of primary root length against time was plotted and linear regression was obtained, yielding the optimum root length (standard) to be used in subsequent cytological experiments.

2.2. Plantlet Regeneration and Determination of Optimum Rooting Media. The seeds of *Dianthus caryophyllus* Linn. cv. Grenadin bought from Yates Company, Australia, were surface-sterilized and germinated on moist cotton wool as previously described. Four-day-old primary roots with a standard length of 11.15 ± 0.33 mm were used to initiate the cultures of this species. The 4-day-old primary roots were excised and immersed in 70% (v/v) ethanol for a few seconds, followed by washing 3 times with sterile distilled water prior to tissue culture initiation. The primary root segments were cultured on MS [2] media supplemented with various combinations and concentrations of plant hormones, such as 0.5–3.0 mg L^{-1} α-naphthalene acetic acid (NAA) and 0.5–3.0 mg L^{-1} 6-benzyl aminopurine (BAP). The media were added with 30 g/L sucrose, pH 5.8 ± 0.1, solidified with 8 g/L agar technical no. 4, and autoclaved at 120°C for 20 minutes. The cultures were maintained in the culture room at 25 ± 1°C with 16 hours light and 8 hours dark for 6 months.

2.3. Morphology of Ex Vitro and In Vivo Grown Plants. Complete *Dianthus caryophyllus* plantlets were transferred to covered vases containing a 1 : 1 : 1 mixture of sand : garden soil : burnt soil and acclimatized in the culture room at 25 ± 1°C with light intensity of 800–1100 lux and a photoperiod of 16 hours light and 8 hours dark for 4 weeks. The plants were watered twice daily with distilled water. The plantlets were subsequently transferred to a greenhouse at 18 ± 2°C with light intensity of 400–1200 lux and a photoperiod of 12 hours light and 12 hours dark, and their growth performance in the natural environment was monitored. The morphological features such as the shape of the leaves, flowers, plant height, and mean leaf diameter of both *in vivo* and *ex vitro Dianthus caryophyllus* were compared to determine any morphological irregularities that might arise due to tissue culture stress or protocols.

2.4. Cytological Analysis on Roots of In Vivo and In Vitro Grown Plants. MS media supplemented with 2 mg L^{-1} NAA were found to be the most optimum media for the induction of roots of *Dianthus caryophyllus*; therefore primary roots obtained from *in vitro* cultures grown on this regeneration media were used throughout the experiment. Newly formed roots were excised from 1-, 2-, 3-, and 4-day-old; 1-, 2-, 3-, 4-, 5-, 6-, 7-, and 8-week-old; and 3-, 4-, and 6-month-old plantlets and preserved overnight in a 3 : 1 ratio of absolute alcohol : glacial acetic acid. The root segments were stained using Feulgen and made into permanent slides, prior to measurements of cellular parameters such as mitotic index (MI), chromosome number, DNA content and C value, mean nuclear and cell areas, and cell doubling time (Cdt) of this species.

The root segments were washed twice with distilled water for 5 minutes and then immersed in 5 M hydrochloric acid (HCl) for 20 minutes, followed by soaking in Feulgen for 2 hours. Feulgen-stained root tips without the root caps were transferred onto glass slides, and added with 1-2 drops of 45% (v/v) acetic acid. The slides were then made permanent based on the quick-freeze method described by Conger and Fairchild [11], and cover slides were mounted on the slides using DPX (Di-N-Butyl Phthalate in Xylene). Visualizations of the cells and chromosomes of *Dianthus caryophyllus* were conducted using a light microscope (Zeiss Axio Scope, Germany) connected to a Sony video camera, supported by VIDAS (Kontron Electronic, Germany).

Three permanent slides with at least 500 cells were observed to determine the mitotic index, which is the percentage of cells that are going through mitosis. The calculation of mitotic index was done based on the following formula:

$$\text{mitotic index (MI)} = \frac{\text{number of cells undergoing mitosis}}{\text{total number of cells}} \times 100, \quad (1)$$

whereby mitosis include cells in prophase, metaphase, anaphase and telophase.

Furthermore, at least 15 cells at metaphase spread were analyzed to determine the mean chromosome number of this species *in vivo* and *in vitro*. Cell doubling time (Cdt) of *in vitro* root meristem was measured from root cells with the highest mitotic index (MI), whereby the root segments were soaked in 0.5 mL of 0.025% (v/v) colchicine for 6 hours. Root segments previously soaked for 1, 2, 3, 4, 5, and 6 hours in colchicine were preserved in a 3 : 1 ratio of absolute alcohol : glacial acetic acid and made into permanent slides, as previously described.

Four-day-old root segments of *in vivo* grown *Dianthus caryophyllus* with a standard length of 11.15 ± 0.33 mm (standard growth) were also subjected to cytological experiments. Cellular parameters such as mitotic index (MI), chromosome number, DNA content and C value, mean nuclear and cell areas, and cell doubling time (Cdt) of *in vivo* grown root meristem cells were measured and compared with those of *in vitro* grown plantlets. The Cdt was measured by plotting the graph of frequency of metaphase (depicted in terms of percentage) against time (duration of exposure to colchicine), which yielded linear regression lines ($y = mx + c$) for both *in vivo* and *in vitro* (Figure 1). The slope of the graph (m) represented the rate of accumulation of cells at the metaphase stage, which would be used in determining the cell doubling time (Cdt) based on Clowes' [12] formula:

$$\text{cell doubling time (Cdt)} = \frac{\ln 2}{m}, \quad (2)$$

whereby m is the gradient of the graph.

2.5. Statistical Analysis. Different concentrations of hormones were assessed using randomized complete block design (RCBD) with 30 replicates to decrease error and enhance accuracy. Statistical analysis was conducted using statistical variance test (ANOVA) and compared using Duncan's multiple range test (DMRT) with the least significant differences at 5% level.

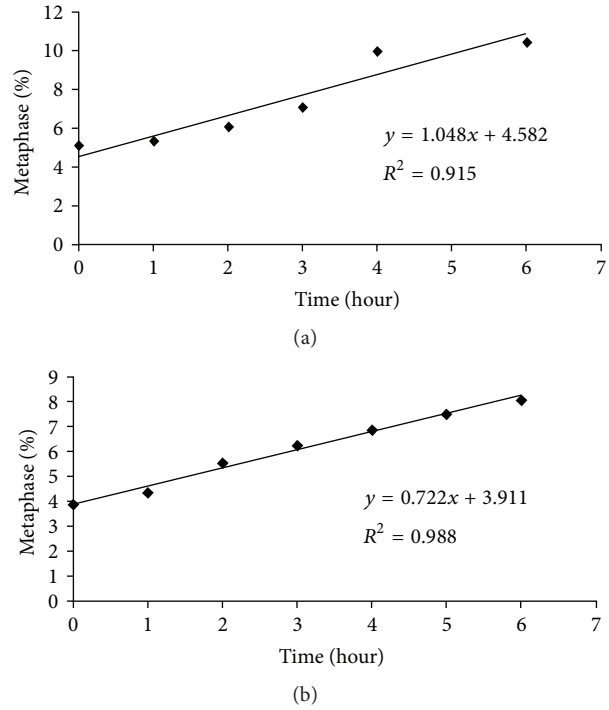


Figure 1: Relationship between percentage of metaphase frequency and duration of exposure to 0.025% colchicine for meristematic cells of *Dianthus caryophyllus* roots grown (a) *in vivo* and (b) on an MS medium supplemented with 2.0 mg L^{-1} NAA.

3. Results

3.1. Plantlet Regeneration and Determination of Optimum Rooting Media. In general, *in vitro* cultures of *D. caryophyllus* primary root segments (with a standard length of 11.15 ± 0.33 mm) on MS media supplemented with different combinations and concentrations of NAA and BAP were found to yield production of callus. White callus was formed when the root segments were cultured on MS media supplemented with 0.5–2.0 mg L^{-1} NAA and combinations of 0.5 mg L^{-1} NAA and 0.5, 1.0, and 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} NAA and 0.5–1.5 mg L^{-1} BAP, 1.5 mg L^{-1} NAA and 2.0 mg L^{-1} BAP, and 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP (Table 1). White and green calluses were also produced from root segments cultured on MS media fortified with combinations of 1.5 mg/NAA and 0.5–1.5 mg L^{-1} BAP, 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, and 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} BAP (Table 1). On the other hand, additions of 2.0 mg L^{-1} NAA and 1.5 mg L^{-1} BAP yielded the formation of compact green callus (Table 1).

Direct root organogenesis was observed from cultures fortified with only BAP (0.5–2.0 mg L^{-1}) and when high concentrations of NAA (3.0 mg L^{-1}) were added (Table 1). Furthermore, indirect root organogenesis was also observed from the callus grown on MS media supplemented with NAA alone (0.5–2.0 mg L^{-1}) and combinations of 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP, 1.5 mg L^{-1} NAA and 2.0 mg L^{-1} BAP, and 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP (Table 1). Production of roots was best achieved on MS supplemented with 2.0 mg L^{-1} NAA (Table 1), which yielded the highest number of roots (100%) and showed formation of secondary roots after as early as 7 days.

3.2. Cellular Behaviour Studies of In Vivo and In Vitro Grown Plants. Determination of standard growth of *D. caryophyllus* primary roots revealed that formation of primary roots was most optimum after 4 days, with a standard length of 11.15 ± 0.33 mm. The rate of root elongation (2.96 mm per day) was also determined from the standard growth graph, which yielded a linear regression line of $y = 2.96x - 1.98$ (data not shown). *In vivo* and *in vitro* grown *D. caryophyllus* root meristems with a standard length of 11.15 ± 0.33 mm were subjected to cytological analysis to determine their mean mitotic index (MI) values, mean chromosome numbers, mean nuclear and cell areas, DNA C values, and cell doubling time (Cdt). The effect of culture duration was also assessed in the current investigation.

It was observed that the mitotic index (MI) values decreased significantly when *in vivo D. caryophyllus* cells entered the tissue culture system, from 43.51 ± 2.14% (*in vivo*) to 41.20 ± 0.79%, 40.23 ± 1.30%, and 37.35 ± 0.40 after 1, 2, and 3 days in culture, respectively (Table 2). On the fourth day, the mitotic index was found to be astonishingly low (32.32 ± 1.55%) compared to that of the *in vivo* plant (Table 2). Interestingly, a significant increase in MI values was recorded after 1 and 3 weeks of culture, with MI values of 39.20 ± 1.54% and 43.77 ± 2.33%, respectively (Table 2). The MI values gradually decreased with increasing culture time,

Table 1: Callus induction and rhizogenesis from root explants of *Dianthus caryophyllus* cultured on an MS medium supplemented with various hormones after 6 months of culture.

MS media + hormone (mg L^{-1})		Rhizogenesis		Callus formation (%)	Colour of callus	Observations
NAA	BAP	%	Mode			
0.0	0.0	NR	NR	NR	N/A	Necrotic
0.5	0.0	57.50[f]	Indirect	20.00[a]	White	Friable callus Roots formed after 8 days
1.0	0.0	100.00[g]	Indirect	100.00[m]	White	Friable callus Roots formed after 7 days
1.5	0.0	100.00[g]	Indirect	90.00[l]	White	Friable callus Roots formed after 7 days
2.0	0.0	100.00[g]	Indirect	100.00[m]	White	Friable callus Roots formed after 7 days
0.0	0.5	NR	N/A	NR	N/A	Necrotic after 6 weeks
	1.0	NR	N/A	NR	N/A	Necrotic after 6 weeks
	1.5	NR	N/A	NR	N/A	Necrotic after 6 weeks
	2.0	NR	N/A	NR	N/A	Necrotic after 6 weeks
0.5	0.5	5.00[a]	Indirect	35.00[d]	White	Friable callus with roots
	1.0	NR	N/A	43.00[e]	White	Friable callus with roots
	1.5	NR	N/A	NR	N/A	Necrotic after 7 weeks
	2.0	NR	N/A	25.00[b]	White	Friable callus with roots
1.0	0.5	10.00[b]	Indirect	55.00[g]	White	Friable callus with roots
	1.0	NR	N/A	82.00[j]	White	Friable callus with roots
	1.5	NR	N/A	20.00[a]	White	Friable callus with roots
	2.0	NR	N/A	NR	N/A	Necrotic after 5 weeks
1.5	0.5	NR	N/A	100.00[m]	White and green	Friable callus
	1.0	NR	N/A	75.00[h]	White and green	Friable callus
	1.5	NR	N/A	80.00[i]	White and green	Friable callus
	2.0	45.00[e]	Indirect	30.00[c]	White	Friable callus with roots
2.0	0.5	NR	N/A	75.00[h]	White and green	Friable callus
	1.0	30.00[d]	Indirect	85.00[k]	White	Friable callus with roots
	1.5	NR	N/A	50.00[f]	Green	Compact callus
	2.0	NR	N/A	30.00[c]	White and green	Friable callus
3.0	1.0	10.00[b]	Direct	NR	N/A	Necrotic after 6 weeks
	3.0	25.00[c]	Direct	30.00[c]	N/A	Friable callus with roots

*Means with different letters in the same column differ significantly at $P < 0.05$ by one-way ANOVA and Duncan's multiple range test.
(NR: no response, N/A: not available).

with the lowest MI value recorded after 3 months of culture (31.83 ± 0.81%). The highest MI value (43.77 ± 2.33%) was recorded after 3 weeks of culture; hence 3-week-old *D. caryophyllus* root segments were used in the determination of cell doubling time (Cdt).

Cell doubling time of *in vivo* grown *D. caryophyllus* root meristems was determined from 4-day-old primary root segments with a standard length of 11.15 ± 0.33 mm, while the Cdt of *in vitro* grown *D. caryophyllus* was determined from 3-week-old root segments (which demonstrated the highest MI value). The Cdt was measured by plotting the graph of the frequency of metaphase (depicted in terms of percentage) against time (duration of exposure to colchicine), which yielded linear regression lines ($y = mx + c$) for both *in vivo* and *in vitro* (Figure 1). Integration of m values (1.05%/hour for *in vivo* and 0.7215%/hour for *in vitro*) into Clowes' [12] formula revealed that *in vitro D. caryophyllus* had a significantly higher cell doubling time (96.07 hours) than *in vivo* plants (66.11 hours).

On the other hand, the chromosome numbers recorded for *in vitro* grown *D. caryophyllus* showed no significant difference compared to *in vivo D. caryophyllus*, with mean chromosome numbers of 29.03 and 29.73, respectively (Table 3). It was also observed that culture time had no significant effect on the chromosome number of *in vitro* grown *D. caryophyllus* (Table 3). Feulgen-stained meristematic cells of *in vitro D. caryophyllus* at various age, showing 30 chromosomes per cell are shown in Figure 2. In contrast, the mean nuclear and cell areas of *D. caryophyllus* root meristematic cells underwent an abrupt change when transferred to *in vitro* conditions, as

Table 2: Comparison between mitotic index (MI) of *in vivo* and *in vitro* grown *Dianthus caryophyllus* root meristems.

Dianthus caryophyllus	MS media + hormone (mg L^{-1})	Age	Mitotic index, MI (Percentage, %)
In vivo	—	4-day-old (standard growth)	43.51 ± 2.14^{f}
In vitro	2 mg L^{-1} NAA	1-day-old	41.20 ± 0.79^{e}
		2-day-old	40.23 ± 1.30^{de}
		3-day-old	37.35 ± 0.40^{c}
		4-day-old	32.32 ± 1.55^{a}
		1-week-old	39.20 ± 1.54^{cd}
		2-week-old	38.67 ± 1.35^{cd}
		3-week-old	43.77 ± 2.33^{f}
		4-week-old	38.62 ± 1.75^{cd}
		5-week-old	38.20 ± 3.24^{c}
		6-week-old	37.31 ± 1.27^{c}
		7-week-old	37.69 ± 0.66^{c}
		8-week-old	38.29 ± 1.87^{c}
		3-month-old	31.83 ± 0.81^{a}
		4-month-old	34.45 ± 0.70^{b}
		6-month-old	33.17 ± 0.78^{ab}

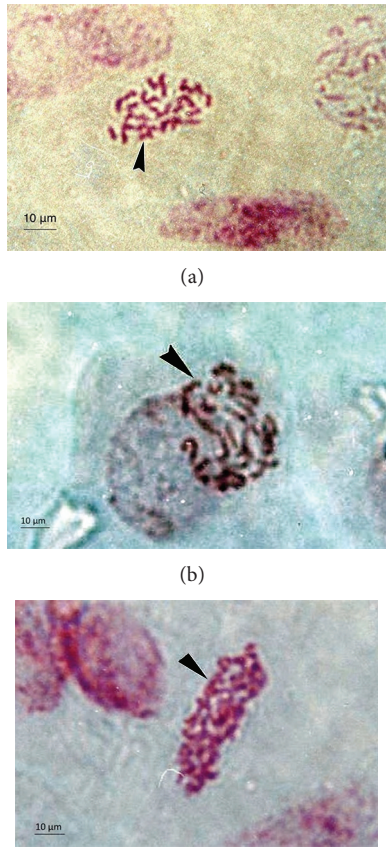
*Means with different letters in the same column differ significantly at $P < 0.05$ by one-way ANOVA and Duncan's multiple range test.

Table 3: Comparison between chromosome numbers of *in vivo* and *in vitro* grown *Dianthus caryophyllus* root meristems.

Dianthus caryophyllus	MS media + hormone (mg L^{-1})	Age	Chromosome number (mean)
In vivo	—	4-day-old (standard growth)	29.73 ± 0.12^{a}
In vitro	2 mg L^{-1} NAA	1-day-old	29.40 ± 0.31^{a}
		2-day-old	28.87 ± 0.29^{a}
		3-day-old	29.67 ± 0.16^{a}
		4-day-old	29.00 ± 0.48^{a}
		1-week-old	29.47 ± 0.40^{a}
		2-week-old	29.87 ± 0.24^{a}
		3-week-old	29.93 ± 0.42^{a}
		4-week-old	29.53 ± 0.51^{a}
		5-week-old	28.80 ± 0.39^{a}
		6-week-old	28.73 ± 0.44^{a}
		7-week-old	28.67 ± 0.36^{a}
		8-week-old	27.93 ± 0.43^{a}
		3-month-old	28.27 ± 0.60^{a}
		4-month-old	28.31 ± 0.69^{a}
		6-month-old	29.00 ± 0.60^{a}

*Means with different letters in the same column differ significantly at $P < 0.05$ by one-way ANOVA and Duncan's multiple range test.

shown by the significant increase of both mean nuclear and cell areas after 1 day of culture (Table 4), with a mean nuclear



(a)

(b)

(c)

Figure 2: Squashed preparation of *in vitro* grown *Dianthus caryophyllus* root tip meristem; showing 30 chromosomes in a cell ((a) 1-week-old, (b) 3-month-old, and (c) 6-month-old). Arrow shows the stained chromosomes in *D. caryophyllus* meristematic cell.

area of $35.66 \pm 0.10\,\mu m^2$ (*in vitro*) and a mean cell area of $165.05 \pm 0.58\,\mu m^2$ (*in vitro*) compared to a mean nuclear area of $26.59 \pm 0.09\,\mu m^2$ (*in vivo*) and a mean cell area of $142.90 \pm 0.59\,\mu m^2$ (*in vivo*). However, the mean nuclear and cell areas were observed to be inconsistent and fluctuated throughout the duration of the tissue culture period (Table 4).

In general, it was found that the nuclear DNA content for both *in vivo* and *in vitro* grown *D. caryophyllus* root meristematic cells had very high percentages of polyploid cells, with a nuclear DNA C value of more than 4.8 C (Table 5). The degree of polyploid cells decreased with culture time, whereby the percentages of polyploid cells were reduced to 61.64% and 63.33% after two and three days of culture (Table 5). However, the percentage of polyploid cells was observed to be generally lower throughout the duration of the tissue culture period although the values fluctuated and appeared inconsistent (Table 5). Furthermore, it was observed that no cells were arrested at the G1 phase, except 2-week-old, 6-week-old, 7-week-old, and 4-month-old cells which showed 1.30%, 1.32%, 0.67%, and 0.68% cells arrested at the G1 phase, respectively (Table 5). It was also observed

Table 4: Comparison between mean nuclear and cell areas of *in vivo* and *in vitro* grown *Dianthus caryophyllus* root meristems.

Dianthus caryophyllus	MS media + hormone (mg L^{-1})	Age	Mean nuclear area, μm^2 (N)	Mean cell area, μm^2 (C)	Ratio (N/C)
In vivo	—	4-day-old (standard growth)	26.59 ± 0.09^{bcd}	142.90 ± 0.59^{def}	0.19^{a}
In vitro	2 mg L^{-1} NAA	1-day-old	35.66 ± 0.10^{efgh}	165.05 ± 0.58^{g}	0.22^{ab}
		2-day-old	30.34 ± 0.09^{bcdef}	124.73 ± 0.44^{c}	0.24^{abc}
		3-day-old	42.37 ± 0.12^{hi}	153.06 ± 0.48^{fg}	0.28^{cd}
		4-day-old	32.30 ± 0.08^{cdefg}	118.94 ± 0.04^{bc}	0.27^{bcd}
		1-week-old	43.95 ± 0.28^{i}	181.48 ± 0.49^{h}	0.24^{abc}
		2-week-old	29.64 ± 0.11^{bcde}	145.58 ± 0.56^{ef}	0.20^{a}
		3-week-old	17.66 ± 0.06^{a}	83.27 ± 0.31^{a}	0.21^{a}
		4-week-old	33.03 ± 0.10^{defg}	106.57 ± 0.30^{b}	0.31^{d}
		5-week-old	30.48 ± 0.11^{bcdef}	129.92 ± 0.54^{cd}	0.23^{abc}
		6-week-old	25.95 ± 0.09^{bc}	131.28 ± 0.56^{cde}	0.20^{a}
		7-week-old	38.21 ± 0.12^{ghi}	142.23 ± 0.59^{def}	0.27^{bcd}
		8-week-old	30.23 ± 0.10^{bcdef}	143.38 ± 0.54^{def}	0.21^{a}
		3-month-old	36.50 ± 0.12^{efgh}	157.14 ± 0.60^{fg}	0.23^{abc}
		4-month-old	36.88 ± 0.12^{fgh}	153.14 ± 0.61^{fg}	0.24^{abc}
		6-month-old	24.53 ± 0.09^{b}	105.27 ± 0.44^{b}	0.23^{abc}

*Means with different letters in the same column differ significantly at $P < 0.05$ by one way ANOVA and Duncan's multiple range test.

Table 5: Comparison of percentage of nuclei in various cell cycle phases between *in vivo* and *in vitro* grown *Dianthus caryophyllus* root meristems.

Dianthus caryophyllus	MS media + hormone (mg L^{-1})	Age	Cell cycle phase (%)			Polyploidy (%)
			G1	S	G2	
In vivo	—	4-day-old (standard growth)	—	1.32^{a}	3.95^{a}	94.74^{ij}
In vitro	2 mg L^{-1} NAA	1-day-old	—	—	—	100.00^{j}
		2-day-old	—	11.32^{f}	27.04^{g}	61.64^{bc}
		3-day-old	—	0.67^{a}	36.00^{h}	63.33^{bcd}
		4-day-old	—	3.27^{bc}	9.15^{b}	87.58^{ghi}
		1-week-old	—	2.01^{ab}	22.82^{f}	75.17^{ef}
		2-week-old	1.30^{b}	17.63^{i}	20.78^{de}	60.39^{b}
		3-week-old	—	11.04^{f}	21.43^{ef}	67.53^{bcde}
		4-week-old	—	1.42^{a}	3.55^{a}	95.04^{ij}
		5-week-old	—	9.09^{e}	19.58^{d}	71.33^{de}
		6-week-old	1.32^{b}	3.97^{c}	9.27^{b}	85.43^{gh}
		7-week-old	0.67^{a}	12.67^{g}	20.67^{de}	66.00^{bcde}
		8-week-old	—	14.00^{h}	43.33^{i}	42.67^{a}
		3-month-old	—	6.08^{d}	13.51^{c}	80.41^{fg}
		4-month-old	0.68^{a}	0.68^{a}	4.73^{a}	93.92^{hij}
		6-month-old	—	18.75^{i}	10.42^{b}	70.83^{cde}

*Means with different letters in the same column differ significantly at $P < 0.05$ by one way ANOVA and Duncan's multiple range test.
(—: absent/no data observed).

that more cells were arrested at the G2 phase compared to the G1 phase (Table 5).

4. Discussion

Observations of mitotic chromosomes under the light microscope are still an informative and rapid method, essential for genomic study [5]. Changes in cell activities can be triggered when cells are transferred from one environment to a different environment [13]. For example, the transfer from *in vivo* to *in vitro* environments can trigger changes of cellular behaviour to occur [14]. Cellular behaviour of a species can be evaluated through cytological studies, such as the determination and comparison of the DNA content,

chromosome count, genetic stability, and cell cycle [15]. Swartz et al. [16] stated that the transfer from *in vivo* to *in vitro* conditions can also affect the genetic constitution of the tissues. Therefore, cellular parameters such as mitotic index (MI), cell doubling time (Cdt), chromosome number, mean nuclear and cell areas, DNA content, and ploidy analysis were determined in the present investigation to elucidate any striking differences that might have occurred as a result of tissue culture procedures.

Mean mitotic indices of both *in vivo* (43.51 ± 2.14%) and *in vitro* (31.83 ± 0.81% to 43.77 ± 2.33%) grown *Dianthus caryophyllus* were quite high compared to *Petunia hybrida* [8] and *Vicia faba* [17] which showed MI values of 11.63 ± 0.26% and 13.82 ± 2.41%, respectively. Mozaffari and Gahan [18] also reported very low MI values for *Pisum sativum* (6.47 ± 1.49%), *Zea mays* (9.52 ± 1.05%), and *Allium cepa* (6.38 ± 2.18%). However, MI values differ subjected to growth conditions, for example *in vivo* or *in vitro*, and undergoing callogenesis or organogenesis. The high MI values of *D. caryophyllus* perhaps indicated that the meristematic cells were actively dividing and had a high regeneration potential. It was also observed that mean mitotic indices of *in vitro D. caryophyllus* were generally lower than in the *in vivo* grown plants, suggesting that the regeneration potential of *in vitro D. caryophyllus* decreased with culture time or the cells were adapting to culture conditions slowly. Abu Shah and Taha [19] also reported lower MI values in root cells of *in vitro* grown *Psophocarpus tetragonolobus* (3.88%) compared to *in vivo* (4.37%) plants of similar species. However, it was found that the mitotic index values fluctuated (although generally lower than *in vivo*) with culture time. A severe reduction in MI values of *D. caryophyllus* was observed after 4 days of culture, from 43.51 ± 2.14% (*in vivo*) to 32.32 ± 1.55%. The drastic reduction was probably due to tissue culture shock that occurred when the cells of *D. caryophyllus* entered the tissue culture system, resulting from the changes in growth environments from *in vivo* to *in vitro*, and could be due to the slow adaptation to the *in vitro* system. The mitotic values gradually increased until after 3 weeks of culture and reached the highest MI value (43.77 ± 2.33%) equivalent to the MI value recorded in *in vivo* plants (43.51 ± 2.14%) and subsequently decreased with culture time. This might be due to the use of growth hormones, specifically the use of auxin (NAA), which contributed to the division and cell elongation [20]. The use of growth hormones had been reported to affect the mitotic index of a species. For example, Das et al. [21] had found that the MI values of parenchyma cells of tobacco pith increased after 6 days of culture when supplemented with IAA and kinetin.

The chromosome numbers of *in vivo* and *in vitro D. caryophyllus* were similar, with approximately 30 chromosomes per cell (Table 3), in agreement with the findings by Carolin [22] in intact plants of the same species. It was also observed that the chromosomal number of *D. caryophyllus* remained stable throughout the culture period (Table 3). However, the root cells of *D. caryophyllus* failed to regenerate multiple shoots despite the chromosomal stability, probably due to the high ploidy level or increased aneuploidy level which in turn could influence the loss of organogenesis capability [23]. In the present study, it was found that the ploidy level of *D. caryophyllus* cells was high throughout the duration of the tissue culture period, with the DNA C value of more than 4.8 C (Table 5). The majority of the cells were found to be polyploid, in both *in vivo* and *in vitro* grown *D. caryophyllus*, although the percentage of polyploid cells fluctuated with culture time (Table 5). The fluctuation of the ploidy level may have an impact on the regeneration potential of the cells and tissues, for example, from 100% to 67.53% after three weeks of culture, which explained the high degree of rhizogenesis that occurred during the culture period.

Growth environments *in vitro* could possibly have enhanced the presence of more polyploid cells in *in vitro* cultured tissues due to the endoreduplication process that occurred within the population of cells, although this process can also occur *in vivo* [24]. Other factors include nuclear restitution or nuclear fragmentation caused by abnormalities such as lagging chromosomes and multipolar spindle, that often result in binucleate or multinucleate cells as well as the occurrence of aneuploidy and reduced chromosome numbers [25]. The balance of auxin and cytokinin in the culture or induction media was also reported to influence the occurrence of nuclear fragmentation and endoreduplication [25]. In the present study, no binucleate or multinucleate cells were observed; therefore it was possible that the high degree of polyploid cells in *in vivo* and *in vitro D. caryophyllus* was caused by nuclear restitution due to abnormal mitoses and chromosomal arrest at the anaphase stage [26], although not proven in the present investigation as no chromosomal aberrations had been observed. An analysis of the results showed that no somaclonal variations had occurred in *in vitro* grown *D. caryophyllus*, where both *in vivo* and *ex vitro* plants appeared morphologically similar. However, further researches are in progress to determine the effects of other growth hormones on genetic stability of *D. caryophyllus* when cultured *in vitro*.

5. Conclusions

Regeneration of *Dianthus caryophyllus* was successfully obtained *in vitro*. The transfer from *in vivo* to *in vitro* conditions was found to have an immediate effect on cell activity of *D. caryophyllus*, where the MI value was found to decrease, while mean cell and nuclear areas increased significantly. However, the mean cell and nuclear areas of *in vitro* grown *D. caryophyllus* appeared unstable and fluctuated throughout the 6-month culture period. Chromosome number ($2n = 2x = 30$) was maintained when *D. caryophyllus* entered the tissue culture system and remained stable throughout the culture period. Ploidy analysis also revealed that *in vivo* grown *D. caryophyllus* contain a high percentage of polyploid cells, which was maintained *in vitro* and throughout the 6-month culture period. Transferring the cells from *in vivo* to *in vitro* environment might have caused the already high percentage of polyploid cells to become more prominent *in vitro*.

Abbreviations

BAP: 6-Benzyl aminopurine
NAA: α-Naphthalene acetic acid.

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