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Assessment Genetic Stability of *Magnolia Grandiflora* L. By Aflp Marker Via Tissue Culture Technology.

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ABSTRACT

This study was done in Tissue Culture & Molecular Biology Labs, Research Centre, College of Science, Duhok University, Iraq, from September 2022 to March 2024, to study the efficient tissue culture protocol for *Magnolia grandiflora* L. and detect the genetic stability of regenerated plants by genetic markers. Lateral buds were used as an explant in this study. Optimal sterilization was obtained with 70% (v/v) Ethanol for 2 min, then explants immersed in (50% v/v) NaOCl for 20 min. At initiation, adding BA at 1.0 mg/l and NAA at 0.05 mg/l to the MS medium gave the highest (2.80 cm) shoot length, medium consist of both BA at 2.0 mg/l with NAA at 0.5 mg/l produced the maximum number (3.00) of leaves. At multiplication, BA at 4.0 mg/l and NAA at 1.0 mg/l gave the maximum shoot length (2.50) cm) with MS medium. A significant difference did not found between MS and WPM media on shoot length. WPM supplemented with NAA (2.0 mg/l) gave the most numbers (4.13) of leaves. WPM was supplied with 0.5 mg/l NAA treatment for developing *in vitro* rooting. Peatmoss with loam in a ratio (1:0.5) (v: v) was used to acclimatize plantlets, resulting in an impressive 80–85% survival rate. Teen AFLP primers combination was used to appreciate the genetic uniformity of regenerated plants. No polymorphism was detected for micropropagated plants as compared with mother plants, proving genetic stability.

***Keywords*:** AFLP, DNA Markers, Genetic Stability, *Magnolia grandiflora* L.

INTRODUCTION

Magnolia (*Magnolia grandiflora*. L.) is an evergreen tree with a pyramid and large white flowers, belonging to Magnoliaceae family, [5, 30]. The genus of magnolia shares about 100 species, produce large, fragrant, bowl-shaped, and creamy flowers, appear from midsummer to early Autumn [3]. The wood of magnolia is used in furniture and much industrial arts. The bark of magnolia contains many compounds available in medicinal (27). Magnolias consider one of an oldest tree species in the world, they may live about 80-120 years [23]. Extraction of Magnolia contained phenolic compounds honokiol and magnolol (Natural products have historically been used as remedies for the alleviation of diseases), Magnolia species are natives to Southeast of Asia, eastern north America and the Caribbean [20], known for their elegant shape and abundant flowers, commonly found in gardens. [7]. Magnolias for their showy flowers and foliage, are valuable ornamental plans [8], they are also grown in order to its timber, which has potential pharmaceutical application. Magnolia is propagated sexually from seeds or asexually by stem cuttings, grafting, layering or budding like most woody plants [1]. [12] have found that seeds from *M. grandiflora* need cold stratification for 90 days in order to achieve a germination rate of about 75%, by layering and stem cuttings may need many years of experience [35]. *In vitro* propagation is a chance to raising the ability to multiply these plants. [4] and [15] report that under long conditions in the *in vitro* process, many factors can cause considerable variation in micro-propagated plants, such as medium culture or growth regulators. Therefore, it is essential to estimate the genetic uniformity for micropropagated plants by using different genetic (DNA) markers. Amplified Fragment Length Polymorphism (AFLP) marker is widely used to assess the stability of many plants.

MATERIALS AND METHODS

1-Explant and sterilization

The lateral bud explants used in this study were collected from (*Magnolia grandiflora* L.), Malta Nursery, Duhok, Iraq, from September 2022 to March 2024. Explants washed under tap water for 1 hour, after washed with sterile distilled water for 5 minutes, under laminar cabinet, explants were soaked with ethanol 70 % for 2 min, then soaked in (2.5% v/v) of NaOCl with tree drops of Tween-20 for 20 min and washed with sterile distill water 3 min, and cultured on MS medium.

2- Initiation Stage

Explants cultured on MS medium supplied with BA (0.0, 0.5, 1.0, and 2.0) mg/l, NAA (0.0, 0.05, 0.1, and 0.5) mg/l, and GA3 at 2.0 mg/l, explants were cultured in the jars with 5 replicates. PVP was added at 0.2 g/l to the

medium to overcome the browning of explants, the cultures placed in controlled condition for 4 weeks, measuring of shoots length and leaves number were recorded.

3- Multiplication stage

At proliferation stage, effects of several concentration of cytokinins and auxins (BA and NAA) and their combinations on shoot multiplication with full MS and WPM were. The plants were cultured on a medium provided with BA (0.0, 2.0, 4.0, and 6.0 mg/l) and NAA at (0.0, 0.5, 1.0, and 2.0 mg/l) with 5 replicate, shoots length and leaves number were recorded after six weeks of culture.

4- Rooting

Shoots about 1.5 cm long produced *in vitro* from lateral bud explants were collected for rooting. WPM (¼ strength) supplied with different concentration of NAA (0.0, 0.1, 0.3, 0.5 mg-1), after 12 weeks of culture, number of roots, roots length was recorded.

5- Acclimatization

The rooted plants after 12 weeks were washed with distill water to separate the agar from roots then putted in a pots with autoclaved mix of (peatmoss & loam) at ratio of (1: 0.5) (V/V) in a sterile boxes, cover with Polyethylene to save the humidity, placed in incubation room and the survival rate was recorded after 30 days.

6- Genetic Stability

To conduct the genetic stability of *Magnolia grandiflora*, the young leaves of the mother and regenerated plants were used to extract the DNA according to [33] method. DNA template of (50 ng) was digested with Psti and Msti restriction enzymes to apply the AFLP marker, and the restriction fragments were ligated by T4 DNA Ligase. The Pre-Selective Amplification then conducted using (P00 & M00) selective primers combination. In total 10 primer combinations were used in selective amplification, the PCR product run on polyacrylamide 8% with 1x TBE buffer at 200 w for about 1.5 h. The gel was stained using the silver staining method, and the bands were scored for analysis.

7- Statistical Analysis

The experiment was arranged using Complete Randomized Design (CRD) with different replicates with each treatment. Data were analyzed and means were compared with each other using Duncan's multiple range test at 0.05.

Results and discussion

1- Explant sterilization

The best results were obtained when lateral bud explants were treated with a solution of 70% (v/v) ethanol for 2 minutes and then explants immersed in 2.5% (v/v) of NaOCl for 20 minutes, in which the contamination percentage was reduced to about (15%).

2-Shoot Initiation

Maximum length shoot (2.80 cm) was produced with MS medium containing BA at 1.0 mg/l and NAA at 0.05 mg/l, while the lowest shoots length (0.10 cm) was obtained with control treatment. [24] mentioned that initiation stage is considered the most crucial part of micropropagation process, because the potential for shoot generation lies with a single bud, and predetermined growth from both axillary and apical buds can still be expressed in culture. Production from pre-existing meristem, such as shoot tips or nodules culture, is the most widely used method *in vitro* with many species [25].

Table (1): Effect of BA, NAA, and combination on shoot length (cm) *at* the initiation of *Magnolia grandiflora* L. lateral bud explant cultured on MS medium after 4 weeks.

BA (mg l^{-1})	NAA (mgl^{-1})				mean effect of BA
	0.0	0.05	0.1	0.5	
0.0	0.10 c	1.00 bc	1.40 a-c	1.50 a-c	1.00 bc
0.5	0.60 bc	1.10 bc	0.60 bc	0.60 bc	0.72 c
1.0	1.60 a-c	2.80 a	1.00 bc	2.00 ab	1.85 a
2.0	2.00 ab	1.50 a-c	1.50 a-c	1.70 a-c	1.67 ab
mean effect of NAA	1.07 a	1.60 a	1.12 a	1.45 a	

*Over all means followed by the same letter(s) within each column or rows do not differe significantly according to Duncan Multiple Range Test at 5% probability.

Typically, BA is used for micropropagation of woody plants [28], it can be applied alone or in combination with NAA [21, 6, 37]. The concentration and type of auxins and cytokinins used depends essentially on plant species, tissues and organs being cultured and the study goals [17]. This result confirmed with that of [18], who observed that an optimal medium for first induction of *Magnolia lucida* was supplied with BA at 1.0 mgl^{-1} + NAA at 0.05 mgl^{-1}.

Table (2) clarified the effect of BA, NAA and combinations on the number of leaves of magnolia lateral buds at initiation. The number of leaves produced was (3.00) when the medium provided with BA at 2.0 mg/l and NAA at 0.5

mg/l. Raising the BA level, enhanced the characteristic of plant the reason may be depends the critical role of cytokinin with synthesis of RNA, protein, enzyme inside the cell, enhancing the growth of buds, break dormancy and apical dominance [1].

Table (2): Effects of BA, NAA, and combinations on leaves number of *Magnolia grandiflora* L. lateral bud explants after 4 weeks of culture on MS medium at the initiation.

BA (mg l^{-1})	NAA (mgl^{-1})				
	0.0	0.05	0.1	0.5	Mean effect of BA
0.0	0.40 c	2.00a-c	2.80a	2.80ab	2.00 ab
0.5	1.20 a-c	1.80 a-c	1.00 bc	2.20 a-c	1.55 b
1.0	1.80 a-c	2.80 ab	1.20 a-c	2.80 ab	2.15 ab
2.0	2.00 a-ca	2.80 ab	1.80 a-c	3.00 a	2.40 a
Mean effect of NAA	1.35 c	2.35 ab	1.70 bc	2.70 a	

*Over all means followed by the same letter(s) within each column or rows do not differe significantly according to Duncan Multiple Range Test at 5% probability.

BA and NAA growth regulators are consider the most prefer choices in woody plants culture. Cytokinin, especially BA, is mostly used in micropropagation, because it is a stable compound, not degraded easily, and is highly effective in breaking the dominance apical [14].

3-Multiplication Stage

Table (3) clarifies the effect of various concentration of BA, NAA, types of media, their interactions on shoot length of *Magnolia grandiflora* L. lateral bud explant at the multiplication stage. BA at 4.0 mg/l and NAA at 1.0 mg/l gave the maximum shoot length (2.50) cm with MS medium. BA alone at 4.0 mg/l produced (1.84) cm, the highest shoot length when compared with other BA concentrations; however, no significant difference was found between MS and WPM media on shoot length. The results indicated that when combined, BA at a high level and NAA at a low level increased shoot length. The favorable influence of BA and NAA may be due to the roles of cytokinin and auxins in supporting cells division, cell elongation, and with regulation of physiological process [36, 12].

Table (3): show the effects of BA & NAA, types of media, and their interactions on shoot length of *Magnolia grandiflora* L. lateral bud explants at the multiplication after six weeks.

Media	BA (mgl^{-1})	NAA (mgl^{-1})				Media*BA	Mean effect of media
		0.0	0.5	1.0	2.0		
MS	0.0	0.25 e	1.50 a-d	1.25 b-e	1.38 a-d	1.09 b	1.46 a
	2.0	1.50 a-d	1.50 a-d	1.25 b-e	2.00 a-c	1.56 ab	
	4.0	1.50 a-d	2.38 ab	2.50 a	1.00 c-e	1.84a	
	6.0	1.25 b-e	1.38 a-d	1.25 b-e	1.50 a-d	1.34 ab	
WPM	0.0	0.88 c-e	1.50 a-d	1.25 b-e	0.88 c-e	1.13 b	1.51 a
	2.0	1.00 c-e	0.75 de	1.75 a-d	2.00 a-c	1.38 ab	
	4.0	1.75 a-d	1.63 a-d	1.75 a-d	2.25 ab	1.84 a	
	6.0	2.00 a-c	1.00 c-e	1.50 a-d	2.25 ab	1.69 a	

Media *NAA	MS	1.13 c	1.69 ab	1.56 a-c	1.47 a-c	Mean effect of BA (mgl^{-1})
	WPM	1.41 a-c	1.22 bc	1.56 a-c	1.84 a	
BA*NAA (mgl^{-1})	0.0	0.56 d	1.50 a-c	1.25 b-d	1.13	1.11 c
	2.0	1.25 b-d	1.13 cd	1.50 a-c	2.00 ab	1.47 b
	4.0	1.63 a-c	2.00 ab	2.13 a	1.63 a-c	1.84 a
	6.0	1.63 a-c	1.19 cd	1.38 a-c	1.88 a-c	1.52 ab
Mean effect of NAA (mgl^{-1})		1.27 b	1.45 ab	1.56 ab	1.66 a	

*Over all means followed by the same letter(s) within each column or rows do not differe significantly according to Duncan Multiple Range Test at 5% probability.

Table (4) summarize the effect of various concentration of BA, NAA, types of media, their interactions on leave numbers of Magnolia lateral bud explant at the multiplication stage. WPM consists of BA at 4.0 mg/l with NAA at 1.0 mg/l, giving the maximum number (4.75) of leaf. WPM showed a significant increase on number (3.52) of leaves comparing with MS medium, which resulted (2.73) leaves/shoots. Results indicated that BA has a positive effect in leaf number, this may related with stimulatory roles of cytokinin in promoting the cell to divide and differentiate [2].

Table (4) summarize the effect of various concentration of BA, NAA, type of media and interaction on number of leaves of *Magnolia grandiflora* L. lateral bud explants at multiplication after six weeks.

Media	BA (mgl^{-1})	NAA (mgl^{-1}) 0.0	0.5	1.0	2.0	Media*B	Mean effect of media
MS	0.0	0.50 h	2.25 e-h	2.25 e-h	3.50 a-f	2.13 c	2.73 b
	2.0	2.75 c-g	3.50 a-f	2.25 e-h	3.25 a-g	2.94 bc	
	4.0	3.00 b-g	4.25 a-d	3.75 a-e	2.50 d-g	3.38 ab	
	6.0	2.75 c-g	2.25 e-h	2.25 e-h	2.75 c-g	2.50 c	
WPM	0.0	1.75 f-h	5.00 a	4.00 a-e	4.50 a-c	3.81 a	3.52 a
	2.0	1.50 gh	2.50 d-g	3.00 b-g	3.25 a-g	2.56 bc	
	4.0	3.75 a-e	2.25 e-h	4.75 ab	4.50 a-c	3.81 a	
	6.0	3.50 a-f	3.25 a-g	4.50 a-c	4.25 a-d	3.88 a	
Media*NAA	MS	2.25 c	3.06 bc	2.63 bc	3.00 bc	Mean effect of BA (mgl^{-1})	
	WPM	2.63 bc	3.25 b	4.06 a	4.13 a		
BA*NAA	0.0	1.13 e	3.63 a-c	3.13 a-d	4.00 ab	2.97 b	
	2.0	2.13 de	3.00 a-d	2.63 b-d	3.25 a-d	2.75 b	
	4.0	3.38 a-d	3.25 a-d	4.25 a	3.50 a-c	3.59 a	

	6.0	3.13 a-d	2.75 b-d	3.38 a-d	3.50 a-c	3.19 ab
Mean effect of NAA (mgl^{-1})		2.44 b	3.16 a	3.34 a	3.56 a	

*Over all means followed by the same letter(s) within each column or rows do not differe significantly according to Duncan Multiple Range Test at 5% probability.

4-Rooting stage

Micro shoots of 2 cm long were used for rooting, and various concentration of NAA, IBA, and different salt strengths were used to induce root formation. WPM at fourth-strength (¼ x) with NAA at 0.5 mg/l resulted a maximum roots number and roots length when compared with other (0.1 & 0.3) NAA concentrations with no root formation. MS medium with the same auxin (NAA) and same concentration did not produce any roots. The ability of Magnolia rooting was affected with salt concentration, the low salt concentration in the medium were known to improve the rooting ability in microshoots. [26]. According to [16], *in vitro* rooting of *Magnolia grandiflora* L. is the most problem in determining this genus in *in vitro* system. Biologically, NAA and IAA are less active than IBA in root formation, IBA producing more roots (29, 31]. Figure (1&2).

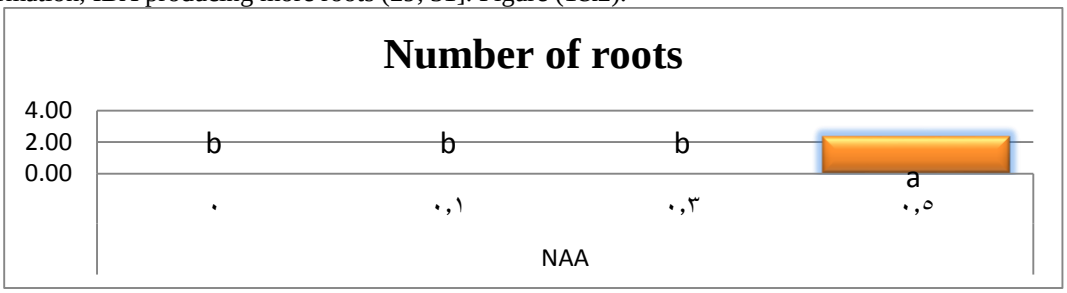

figure (1): effect of NAA on roots number of *Magnolia grandiflora* cultured on fourth-strength (¼ x) WPM after 12 weeks.

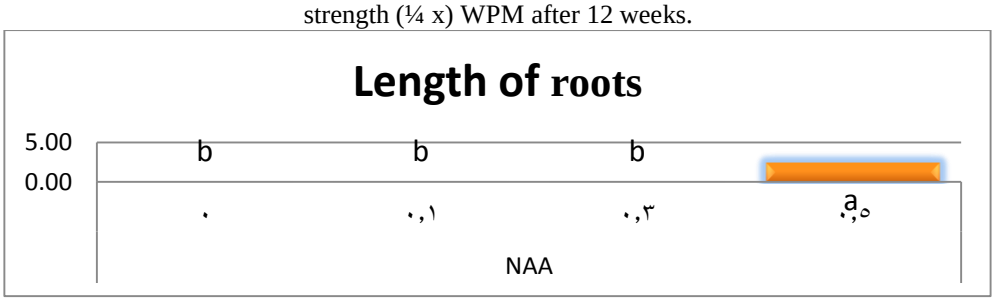

figure (2): effect of NAA on length of roots of *Magnolia grandiflora* cultured on fourth-strength (¼ x) WPM after 12 weeks.

5-Acclimatization

Rooted plantlets were moved from previous cultures to a greenhouse environment after 12 weeks. Plants were cleaned with distilled water, then putted in pots filled with a mix of an autoclaved peat moss, loam (1:0.5) (v: v), then covered by polyethylene to preserve the humidity. The survival rate was 80-85. Low control of water loss from plantlets is considered a challenge that affects the survival rate and growth of plant cultured after transplanting and moving them to photoautotrophic conditions [10].

6-Genetic Stability

In this study, thirty-two AFLP primers were used to confirm genetic stability of micropropageted *Magnolia grandiflora* plants, but only ten amplified products were gave a clear and successful combinations. A total of 210 bands produced using (Pst) and (Mse) primers, run on Polyacrylamide gel 8% electrophoresis at 150-220 v/cm for 1.5h. The band molecular weights ranged from 100 bp to 3000 bp. All amplified fragments produced were monomorphic. The results indicated that micropropagated plants are genetically stable at the assessed genomic regions. Figure (3&4).

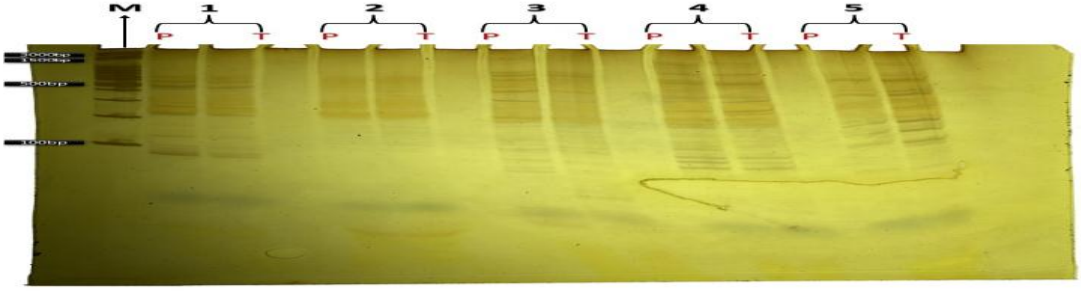

Figure (3): amplification of AFLP profile, generated from DNA of mother and regenerated plants of *Magnolia grandiflora* L. between (Pstl) primer and (Msel) primer. (M) marker (100-3000 bp) run on 8% Polyacrylamide

gel. P. Parent, T. Tissue culture plan
1- P 114 + M 291.
2- P 107 + M 95.
3- P 294 + M307.
4- P 294 + M 291.
5- P 100 + M 82.

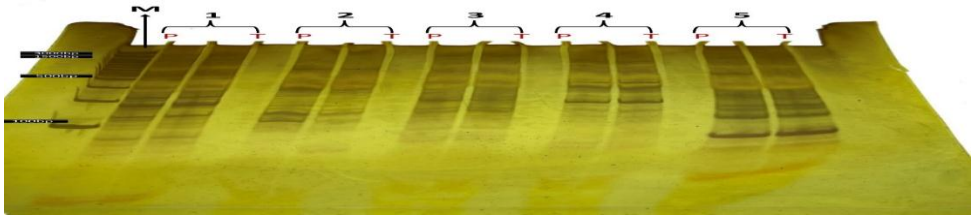

Figure (4): amplification of AFLP profile, generated from DNA of mother and regenerated *Magnolia grandiflora* L, between (Pstl) and (Msel) primers, (M) marker (100-3000 bp molecular weight) run on 8% Polyacrylamide gel. P. Parent, T. Tissue culture plant.
1- P 107 + M 82.
2- P 100 + M 307.
3- P 294 + M 82.
4- P 100 + M 291.
5- P 107 + M 182.

According to [22], the stability of regenerated plants may be affected under various factors, such as PGRs, the composition of media, and stress during micropropagation, which may cause variations *in* regenerated plants. Thus, it is proof that genetic stability is essential for regenerated plants. AFLP marker was used in this study to detect the genetic fidelity of micro-propagated plants after two years of subcultures. The amplification products were monomorphic for regenerated and mother plants, leads that no mutation founds and plants are genetically stabile using the protocol of micropropagation. Our results are consistent with that of previous studies [13, 34, 22].

Conclusions:

Using WPM have significant effect on multiplication and rooting of *Magnolia grandiflora* L. NAA at low levels did not produced roots. Axillary buds as explants in vitro increase the likelihood of genetic stability of regenerated plants. Using of DNA markers (AFLP) is sufficient method to detect the genetic stability of micropropagated plants.

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تقييم الأستقرار ألوراثي للمغنوليا *Magnolia grandiflora* L. بواسطة العلامة AFLP عن طريق زراعة الأنسجة

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الخلاصة

نفذت هذه الدراسة في كل من مختبري زراعة الأنسجة النباتية و مختبر الموليكيولر بايولوجي مركز أبحاث كلية العلوم في جامعة دهوك خلال فترة أذار 2022 ولغاية شهر نيسان 2024 ، لأنشاء بروتوكل فعال لزراعة الأنسجة لأنبات ألماغنوليا والكشف عن الأستقرار الوراثي للنباتات المتجددة. أستخدمت البراعم الجانبية كجزء نباتي خلال التجربة. أظهرت نتائج التعقيم بأن أستخدام كحول الأيثانول بتركيز (70% حجم/حجم) لمدة 2 دقيقة ثم غمر الجزء النباتي في محلول الكلوركس (NaOH) بتركيز 2.5% لمدة 20 دقيقة كانت أفضل معاملة للتعقيم. في مرحلة النشؤ تم الحصول على أفضل طول للفرع النباتي (2.80) سم عند أضافة كل من BAبتركيز (1.0) ملغم/لتر و NAA بتركيز (0.05) ملغم/لتر الى وسط ألزراعة MS كما تم الحصول على أكبر عدد من ألأوراق (3.00) باضافة كل من BA بتركيز 2.0 ملغم/لتر و NAAبتركيز 0.5 ملغم/لتر. في مرحلة التضاعف ، انتجت أفضل طول (2.50)سم للفرع النباتي عندما تم تجهيز الوسط بكل من BAبتركيز 4.0 ملغم/لتر و NAA بتركيز 1.0 ملغم/لتر ولم يكن هنالك فروقات معنوية بين وسطي ألزراعة MS & WPMعلى طول الفرع النباتي. كانت وسط الزراعة WPM (ربع القوة) أفضل وسط لنمو و تطور الجذور عندما تم تجهيزه ب 0.5 ملغم/لتر NAA. في مرحلة الأقلمة تم زراعة النباتات في خليط من البيتموس و اللوم بنسبة حجمية (0.5:1) حيث بلغت نسبة النجاح 80–85 % . تم أستخدام اثنان وثلاثون علامة من AFLP للكشف عن الأستقرار ألجيني بين نبات ألأم والنباتات المنتجة بالأكثار الدقيق وأظهرت ألنتائج بأن هناك أستقرار جيني حيث لم تظهر أختلافات بين نبات ألأم والنباتات المنتجة.

الكلمات المفتاحية: العلامة AFLP ، الاستقرار الوراثي ، العلامة DNA ، Magnoli grandiflora L .