*Egypt. J. Chem.* **Vol. 65**, No. 6. pp. 521 - 533 (2022)

# Egyptian Journal of Chemistry

**http://ejchem.journals.ekb.eg/**

## Characterization Of Two Daffodils Genotypes Using RAPD Markers Under Micropropagation.

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**Abstract**

Two daffodil genotypes were used in a comparative study; *Narcissus tazetta* var. Chinensis and *Narcissus tazetta*cv. Wave. MS medium with different additives were used: MS free as control, MS with2,4-D for callus induction (indirect propagation), and MS with TIBA + IBA and activated charcoal for direct propagation, twin scale was used for explant inoculation, different sub-culturing times and acclimatization were applied. Four primers were used for RAPD-PCR, beside cytological examination for native bulbs of the two daffodils.

Experiments revealed that the regeneration of *Narcissus* showed less than moderate genetic stability to the number of subcultures, media content and through acclimatization; generally *N. t.* cv. Wave had more growth vigour than *N. t.* var. Chinensis. All measured characters revealed that MS medium with 2mg/L 2,4-D provide the chance for more callus induction, whereas MS with 1mg/L IBA + 1mg/L TIBA in addition to 1gm/L of activated charcoal gave more opportunity for explants to be vigor in leaves and roots number as well as length. The profile of RAPD-PCR analysis revealed some changes with differ in medium additives, subculture and acclimatization, reflecting that the two *Narcissus* genotypes have less than moderate genetic variations under three media conditions and conclude that the two genotypes of *Narcissus* have genetic stability under studied conditions. The two genotypes had different chromosome number demonstrated with cytological examination.

*Key words*:Daffodil; Micropropagation; RAPD-PCR; Cytological Examination; Acclimatization.

## Introduction

In Egypt, Daffodils (*Narcissus*) bulbs where they resides from Pharaohs era belong to the Amaryllidaceae family; they flourish in the northern shores during winter season. Remains of *Narcissus* bulbs were found on the neck of the mummy of Ramses II [1]. Amaryllidaceae family is characterized by two genera in Egypt, *Narcissus* and Pancratium [2].

In last decades increasing water soil content increased caused a serious decay in *Narcissus* bulbs. Therefore, *Narcissus* almost disappeared under the pressure of tourism and ordinary cultivation in north coast of this country [1], as well as Juan-Vicedo *et al.*,[3], found *Narcissus* sp. has many endemic, rare and/or endangered species in North Africa and Iberian Peninsula.

*Narcissus* sp. are polymorphic and this genus is sorted as a classical model to study style polymorphism and floral evolution [4,5]. Since the origin and locations of *Narcissus* sp. were close to each other, vast hybrid groups have been regularly arising for thousands of years through natural hybridization. Moreover, the *Narcissus* L. species chromosome number had an abundant variation fluctuating from 2n = 14 to 46, aneuploidy and triploidy frequently happen in natural [6,7]. Consequently, the boundaries of *Narcissus* sp. genetic relationships and species are very complex and persisted unresolved. It is well-known that several earlier cytological studies have been concentrated on Chinese *Narcissus*[8,9], but there is a scarcity of records for the other *Narcissus* species.

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DOI: 10.21608/EJCHEM.2021.107409.4930

Due to high existence of natural hybridization, the classification of *Narcissus* sp. still remains indistinct, and the traditional classification has been based on morphological features [10].

*Narcissus* production faces a number of challenges such as infections , slow vegetative rate and sexual breeding [11], therefore yield expansion programmes hindered with slow reproduction [12]. A lot of efforts was prepared to increase vegetative propagation speed by twin scaling and chipping [13], as well as by *in vitro* micropropagation [3,14-15-16-17-18-19-20-21].

Intense breeding programs of *Narcissus*have led to over 30,000 recorded cultivars daffodils [22], presenting powerful genetic pool and diversity for that genus [23]. Patterns of genetic diversity information can offer vision into the evaluation and conservation practice of the germplasm properties (24-25). Analyzing methods for plant genetic diversity, were advanced recently, whereas generally based on DNA markers, morphological characteristics, isozymes, and seed proteins [26]. Also genetic polymorphisms have been employed to identify.

Numerous DNA markers methods have been used to explore genetic relations among accessions in *Narcissus* [27]. The long generation cycle of *Narcissus* (from *in vitro* plantlets to flowering) take about 4–5 years, the possible morphological variants of flower traits can only be identified at late developing stages. Hence early detection of genetic variability is of a great significance in a breeding program [28].

This study aims to use two *Narcissus* genotypes (*Narcissus tazetta* var. Chinensis and *Narcissus tazetta* cv. Wave). Three types of medium additives were used: MS free as a control, MS with 2mg/L 2,4-D for callus induction (indirect micropropagation) and MS with 1mg/L TIBA + 1mg/LIBA + 1gm/L Activated Charcoal (direct micropropagation).Twin scale method was used for explant inoculation, different sub-culturing times and acclimatization were applied. Besides RAPD markers and cytological examination of the two daffodil genotypes have been studied.

## Materials And Methods

### Plant material:

About two years old bulbs were collected of two daffodil genotypes, *Narcissus tazetta* var. Chinensis and *Narcissus tazetta* cv.Wave were used in this experiment as explant.

### Media:

Three types of medium additives based on MS medium with 3 different additives[29] (M1, M2 and M3) for each genotype of Chinensis *Narcissus* were used as in table (1).

Table (1): Media types for *Narcissus* culture.

| Name | Components |
|---|---|
| M1 | MS medium supplemented with 30 gm/L sucrose + 7 gm/L agar (PH 5.8), this medium was free from hormones |
| M2 | MS medium supplemented with 2mg/L 2,4-D (2,4-dichlorophenoxy acetic acid) + 30 gm/L sucrose + 7 gm/L agar |
| M3 | MS medium supplemented with 1mg/L IBA (indole-3-butyric acid) + 1mg/L TIBA (TriiodobenzoicAcid) + 30 gm/L sucrose + 7 gm/L agar in addition to 1gm/L of activated charcoal |
| Notice | All media types were autoclaved at 121 °C for 20 minutes. (PH 5.8). |

### Bulbs and explants Sterilization:

Sterilization was carried out according to Abdel-Rahman *et al*., [17], firstly, healthy two years old *Narcissus* bulbs were chilled for two weeks at refrigerator, after that bulbs were carefully cleaned under running tap water, then removing outer scales and roots with keeping basal plate intact. The next steps were carried out as in (table 2).Subculture was done after each 4 weeks of culture initiation, cultures were incubated at 18 ± 2 °C, for 16 hours photoperiod/day or subculture was done for 2 and 4 time. Samples from second, fourth subculture and native bulbs were taken for RAPD analysis.

Table (2): Sterilization protocol for *Narcissus* culture bulbs.

| | **Outside cabinet** | **Inside cabinet** |
|---|---|---|
| **1** | Cleaned bulbs soaked in water with soap + clorox for 30 minutes | Bulbs were submerged in 70% ethanol + few drops of Tween 20 for 1 minute (wash once) |
| **2** | Washing with running tap water for 1 hour | 20% Clorox + few drops of Tween 20 for 5 minutes (wash twice) |
| **3** | Soaking bulbs in 70% ethanol for 10 minutes | 0.1% Mercuric Chloride for 1 minute (wash three times) |
| **4** | Soaking bulbs in 2% $HgCl_2$ for 10 minutes | 9% $H_2O_2$ for 1 minute (wash once) |
| **5** | Spray the bulbs with 70% ethanol | |

**Culture establishment:**

- Bulbs of both genotypes (var. Chinensis and cv. Wave), each were handled by the same way and under the same conditions as separate two groups. Ninety bulbs were used for culture initiation from each genotype.

- Bulbs were divided longitudinally into three or four parts depending on the bulbs size whereas twin-scales method was used as explant for culture inoculum.

- Sixty of ninety bulbs were cultured on callus induction medium MS + 2mg/L 2,4-D (M2) for indirect regeneration and incubated in dark conditions at 18 ± 2°C.

- After 3-4 weeks, calli were divided into two groups (a) first group was subcultured on the same medium (M2) and transferred to light condition and (b) second group was transferred to MS free (M1) as control under light condition.

- The last third (30 bulbs) of explants from each genotype were cultured on medium containing 1mg/L IBA + 1mg/L TIBA in addition to 1gm/L of activated charcoal (M3) as direct regeneration, and kept in light condition.
- Subculture was done each 4 weeks and kept at 18 ± 2°C in 16/8 h day/night photoperiod.
- Samples from regenerated bulblets were taken after two and four subculturing and kept in freezer for the RAPD technique.

**Acclimatization:**

The plantlets were taken out from the jars,washing agar away from the roots under running tap water, then the plantlets were cultured into sterilized plastic cups (10×10 cm) containing (1 peat: 1 perlite) mixture. Each cup was irrigated with distilled water every 3 days for 6 weeks. The potted plantlets were initially maintained inside the culture room conditions (24°C) for 6 weeks and later transferred to green house (33 ±1°C) conditions for 8 weeks.

**Molecular studies material:**

Ten samples were chosen from each genotype, classified into three categories as following: three types of media (M1, M2, M3 as in table 1) each explant had different times of sub-culturing (twice S2)and four times (S4) and acclimatized (A), besides control, control sample was taken from healthy native bulb without any culturing in media or any other treatment (table 3).

Table (3): Samples used in RAPD studies for the two genotypes of *Narcissus*.

| **No.** | var. chinensis | Type of medium | **cv. wave** | Type of medium |
|---|---|---|---|---|
| **1** | Control | - | Control | - |
| **2** | S2 | M1 | S2 | M1 |
| **3** | S4 | | S4 | |
| **4** | A | | A | |
| **5** | S2 | M2 | S2 | M2 |
| **6** | S4 | | S4 | |
| **7** | A | | A | |
| **8** | S2 | M3 | S2 | M3 |
| **9** | S4 | | S4 | |
| **10** | A | | A | |
| **Total** | 10 | | | |

**Extraction of DNA:**
The genomic DNA was extracted from fresh leaves samples (ten samples from each medium (table 3) collected from the two *Narcissus* genotypes and from the three media types (M1, M2 and M3) after different subcultures times S2 and S4) and after acclimatization (A),besides control sample which was taken from healthy native bulb without any treatment with EMS according to the protocol of Biospin plant genomic DNA extraction Kit (Bio basic).

- **PCR- Amplification and RAPD Analysis:**

The amplification reaction had performed in a 25µl reaction mixture including 3µl of primer, 2µl of genomic DNA, 1.5 units of Taq DNA polymerase and 200 mM of each dNTPs,2.5µl of 10x Taq DNA polymerase reaction buffer. The subordinate PCR software had exercised in a cycler on thermal DNA (PTC-100 PCR version 9.0-USA). Premier denaturation at 94 ° C for 5 minutes, subsidiary by 35 cycles of 94 ° C for 30 seconds, 42 ° C for 90 seconds. For annealing temperature, 72 ° C for 90 seconds. The extension at 72 ° C for 2 min. The products had detached by RAPD-PCR on 1.5% agarose gels in 1x TAE buffer and disclose by staining with ethidium bromide approbate to Sambrook *et al.*,[30], and Rashad *et al*.,[31].

The output by RAPD- PCR had discrete on 1.5% agarose gels in 1X TAE buffer and disclosure by staining with ethidium bromide approbate to [30]. DNA ladder 100 bp had hired and PCR products had visualized by UV-transilluminator and photographed by gel documentation system, Biometra - Bio Documentations, the amplified bands had recorded as (1) for existence and (0) for the absence of all samples genotypes according to gel analyzer protocol. It had a group of four random 10-mer primers (Table 5and 6) applied in the detection of multiple forms between genotypes. These products synthesized in RAPD-PCR were performed according to procedures presented by Williams *et al.*,[32] with minor modifications.

**Cytological Examination**

- Native *Narcissus* bulbs from the two genotypes were soaked in tap water for 24 hours, then germinated on moistened filter papers in petri dishes at room temperature.

- Primary roots excised at 1.5-2.5 cm long during 8-9 am, then pretreated with 0.005 M 8-hydroxyquinoline for 3 hours at 18 c°.

- Root tips rinsed in distilled water for 10 minutes and fixed in Carnoy,s solution ethanol: glacial acetic acid (3:1) for 24 hours at 4 C° in refrigerator, then roots were washed using distilled water and stored in 70% ethanol.

- Stored roots tips were squashed by tapping them in a drop of 2% acetocarmine stain on slides providing background staining [33].

- After removing the coverslip, slides were air dried in room temperature, coverslips mounted with DPX mounting. Slides were examined and photographed by digital camera.

## Results And Discussion

**Micropropagation:**

***Narcissus tazetta* var. Chinensis**

The calli formed after 3-4 weeks of culture initiation on M2 shown in fig.1A,and aftertransferring to light in both M1(control) and M2for15 days, some embryos-like began to appear (fig.1B). Within 3 weeks emerging of greenbuds were appeared (fig 1C). After a month, the first adventitious growth budsbegan to swallow.
Tiny bulb-like structures appeared at the base of the leaves after about two months (fig. 1D-F), four subcultures were done. In this study twin-scale successfully induced callus, somatic embryogenesis, and organogenesis from both *Narcissus* explants where similar results were found by many studies [17,20,34,35].
Dissimilar foundation by Ferdausi, *et al.*,[20]that twin-scale explants totally failed to induce callus on MS free medium for *Narcissus pseudonarcissus* cv. Carlton.

While, in case of (M3) medium bulblets of both genotypes emerged after about a month (fig. 2A), roots of these explants emerged early and it was obvious that they were more vigor in explants regenerated in (M2) medium and those found to be more potency than in control explants (M1) (fig. 2B) (table 5). Many studies found that twin-scale have the maximum success rate [17,20,36,37].

**Fig. (1)** (A) Callus formation of *N.t.* var. Chinensis on M2. (B) Buds emerging from calli on M2 (C) Separating buds. (D-E-F) Bulblets development.

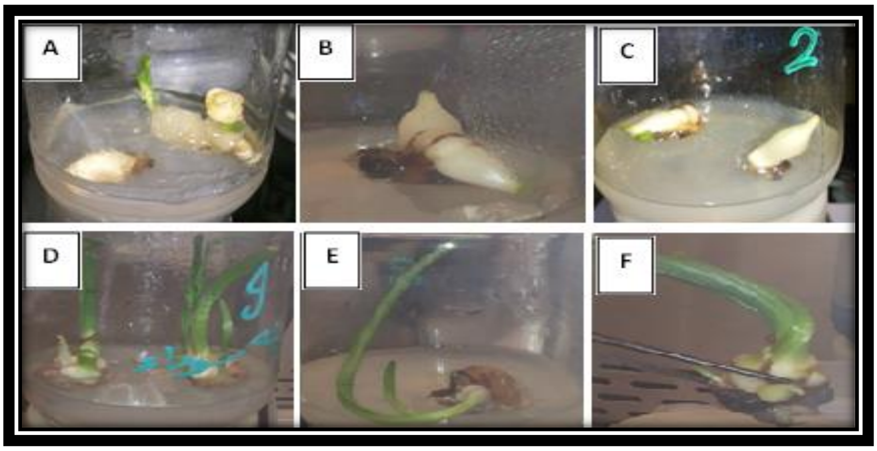
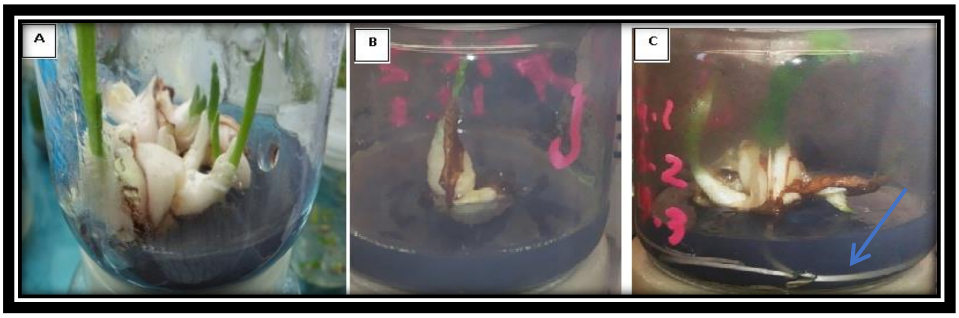


**Fig. (2)** *In vitro* micropropagation of *N.t.* var. Chinensis on M3.(A) Cluster of young bulblets, (B) Single bulblet, and (C) Root formation.



***Narcissus tazetta* cv. Wave**

Generally, the results of *Narcissus tazetta* cv. Wave explants showed more growth vigor than explants from *Narcissus tazetta* var. Chinensis (fig.3). Explants on (M3) media showed better bulblets size, number, leave length, leave number, also roots have obvious vigor in length and thickness (fig. 3- table 5).While explants of both (M1) and (M2) medium slowed less vigor than (M1) as in fig. (4), subcultured bulblet on Wave genotype on control showed strong growth than bulblets cultured on (M2) especially bulb size and roots (fig. 4 and table 5).

**Fig. (3)** *In vitro* micropropagation of *N.t.* cv. Wave on M3. (A) One bulb divided into three parts after three weeks. (B, C) Buds emerging in great number. (D-E) Bulblets development. and (F) New single plantlets.

Results of this study showed that media containing 1g/L of activated charcoal has recorded the most desirable expectations for both Narcissus tazetta genotypes. Many authors are in agreement with this results for in vitro regeneration in some bulbous plants, Bachetta et al., [38] found that AC was associated to a higher bulb size in Lilium sp., and Nhut et al., [39] had record an enhancement of shoot regeneration in L. longiflorum explants. As well as Steinitz and Yahel, [40] found N. tazetta bulblet production could be stimulate initialization culture by AC and N. tazetta "Chinensis" bulblets regeneration [17]. Juan-Vicedo et al., [3] found that addition of AC with 2g/L had no effect with five Narcissus sp.

With exact fine network of pores and large inner surface area (on which various substances can be adsorbed) is frequently used in In vitro propagation to increase cell growth and development. Also, it shows a critical role in micropropagation, somatic embryogenesis, orchid seed germination, stem elongation, rooting, and bulb formation [41].

AC promotary effects on morphogenesis stimulated by its irreversible adsorption of inhibitory compounds in the culture medium and substancially decreasing the phenolic exudation, toxic metabolites and brown exudate accumulation.

AC promote growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene [41]. AC may be regularly relief certain adsorbed products, such as growth regulators and nutrients which become available to plantlets.

**Effects of different culture conditions on yielding plantlets**;

Data of this study concluded that MS medium stimulate roots formation of plantlets and similar findings on root development were reported previously in *Narcissus*[17,20,36,37], this is in agreement with our data in (4) revealed that addition of any plant growth regulators to culture medium of *Narcissustazetta* as in M2 and M3 increased number and length of both leaves and roots, also there was apositive relationship between number of subculture with number and length of leaves and roots in both genotypes under studies in comparative with M1 results.

Regarding the medium level, M3 with Wave genotype gave the highest value in all characters specially with increasing subculture number where we found that four times (S4), it was 10.2, 6.8 give rise to an increasing in the number of leaves and roots up too, and 9.7 cm, 14.1 cm in length of leaves and roots respectively.

While, in case of M3 medium with *Narcissustazetta*genotypesex plants showed the highest value in number of roots and leaves length (6.1, 5.9 cm) under (S4), while the highest value of number of roots and leaves length found to be 8.4,8.9 cm respectively also in (S4) but in M2(MS+1mg 2,4-D).

With regard of the number level/explant, M2 (MS+2mg/L2,4-D) produced the highest number of bulblets/explant in *Narcissustazetta*genotypes specialize with fourth subculture (7.9) followed with the same medium under second subculture (6.5) comparing with the other media. While, M3 (MS+1mg/L IBA+1mg/L TIBA) revealed the highest number of bulblets/explant in Wave genotype specially with subculture fourth times (9.8) followed by the same medium under subculture twice (8.8) comparing with the other media.

Growth regulators used in this study were auxins such as 2,4- dicholrophenoxy-acetic acid (2,4-D), indole butyric acid (IBA), and TIBA as cytokinins. Hence auxins are involved in cell division and elongation, differentiation of vascular tissue, root formation and rhizogenesis, inhibition of auxiliary bods and growth embryogenesis [42,43,44]. As well as Abu Taleb *et al.*, [16] studied the effect of growth regulators (auxins and cytokinins) on *Narcissustazetta* var. italicus and found that type and concentration of growth regulator are necessary for callus induction where this differences dependent on the source of their explant.

Plant growth regulators (PGRs) are essential for cell growth and differentiation [43,45] while auxins higher concentration frequently facilitate the callus formation. The presence of growth regulators was also necessary for callus differentiation; similar findings showed that no differentiation was observed in MS basal medium according to absence of PGRs in *N. pseudonarcissus*[15]. Whereas, Rahman *et al.*,[17] add 2,4-D auxin for *N. tazetta* var. Chinensis micropropagation and Ferdausi, *et al.*,[20] found that the addition of NAA (naphthalene acetic acid) as auxins for in vitro

**Fig. (4)** *In vitro* micropropagation of *N.t.* cv. Wave on control M1. (A, B)-Bud emerging. (C) Bud development. (D) Single bulbletand (E, F) Separating plantlets with strong root.

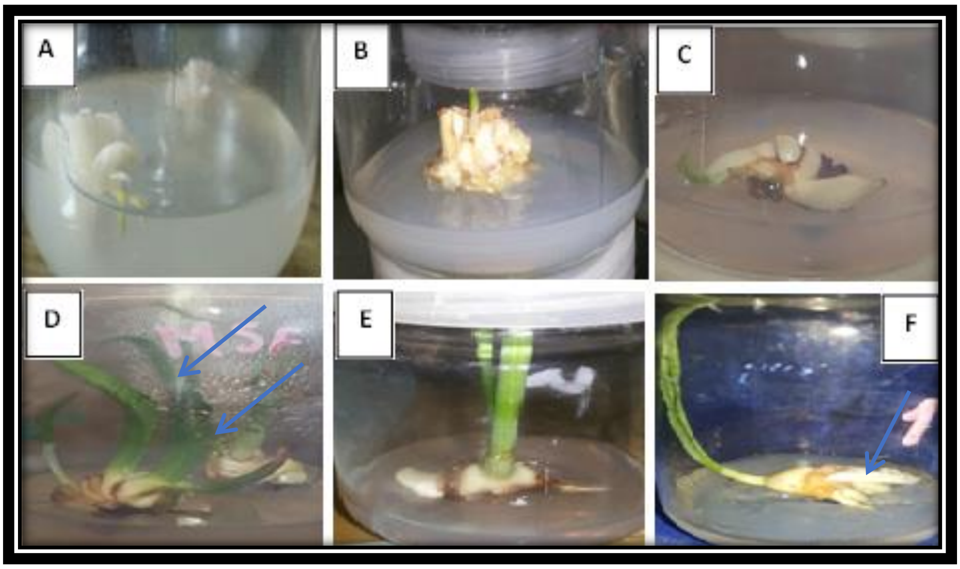



Table (4) Effect of different medium and subculture times on leaves and roots number and length in addition to number of bulblets in two *Narcissus*genotypes.

| | | No. ofleaves/explant | | No. of Roots/explant | | leaves length [cm]/explant | | Root Length [cm] /explant | | Number of bulblets/explant | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genotype** | **Medium** | **S2** | **S4** | **S2** | **S4** | **S2** | **S4** | **S2** | **S4** | **S2** | **S4** |
| | **M1** | **5.5** | **5.8** | **3.5** | **3.7** | **3.3** | **5.2** | **5.8** | **8.2** | **4.2** | **5.5** |
| **Chinensis** | **M2** | **7.1** | **8.4** | **3.4** | **3.5** | **2.5** | **4.9** | **7.3** | **8.9** | **6.5** | **7.9** |
| | **M3** | **4.8** | **6.9** | **4.9** | **6.1** | **4.7** | **5.9** | **6.4** | **7.9** | **5.1** | **6.2** |
| | **Control M1** | **5.8** | **6.3** | **3.7** | **3.9** | **5.2** | **8.1** | **5.5** | **7.5** | **5.0** | **5.8** |
| **Wave** | **M2** | **8** | **8.8** | **3.6** | **3.8** | **4.6** | **6.1** | **7.8** | **10.6** | **7.4** | **8.1** |
| | **M3** | **8.4** | **10.2** | **5.2** | **6.8** | **5.5** | **9.7** | **8.3** | **14.1** | **8.8** | **9.5** |

These results are in arrangement with Islam et al. [46] who settled that growth regulators have a main effect and a regulatory role on callus and root growth. Wiktorowska *et al.*,[47]cited that auxins have a significant role in callus induction and their action could be facilitated by minor concentrations of cytokinins. Taleb *et al.*, [16], study showed that if level of auxins is higher than cytokinins, was the most appropriate medium for callus induction and growth resulting from bulb explants.
Ferdausi *et al.*, [20], study on *Narcissus pseudonarcissus* cv. Carlton found that callus was induced mostly from MS medium comprising high auxins concentration (20 mg/l NAA (naphthalene acetic acid)), while medium with low auxins (4 mg/l NAA) on MS medium provided bulblets with both white and green leaves.

Anbari *et al.*,[37], studied micropropagation of *Narcissus papyraceus* cv. Shirazi and found that explants cultured in Nitsch and MS media having different concentrations of growth regulators produced highest number of regenerated plantlets in MS containing 0.5 mg/l GA3, 1.6 mg/l BAP and 1.6 mg/l 2,4-D. While, somatic embryos induced were multiplied by transferring them to MS free. Santos et al., [48] studied conditions for micropropagation of *Narcissusasturiensis* bulb, using twin-scales as primary explants cultured in MS accompanied with BA (1.99 mg/l), IBA (1 mg/l) and BA (5.99 mg/l, NAA (0.12 mg/l.

Previous studies showed that 2,4-D presence in media for *Narcissus* sp. proved to be suitable for induction of somatic embryogenesis in *Narcissus* and other genera of this Amaryllidaceae family as (49-50-51).El Tahchy*et al.,* (15), found that increasing 2,4-D concentration will decreasing the survival ratio of the explants, showing a toxic effect. Effects of each BA and 2,4-D alone were not significant but the adding of 2,4-D and BA together had a positive effect on callusing root and bulb formation.

In recent experiment medium M3 which containing IBA and TIBA (2,3,5-triiodobezoic acid) gave the best results, it considered as auxin transport inhibitor, this was similar to many previous studies [17,18,52,53]. In these experiments adding TIBA increased shoot formation significantly from twin scale explants, as well as it is a tools to enhance branching, also play a role as an inhibitor of polar auxin transport that has been found to reduce apical dominance and increase branching in other species [18].

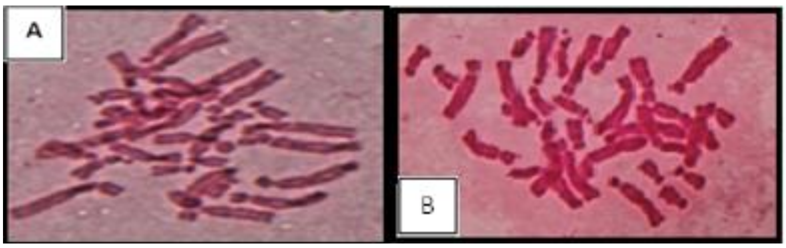
The plant growth regulatorIBA (indole-3-butyric acid) that plays a role in plantlets rooting [18], they found thatthe newly formed shoots were rooted in vitro on medium with 0.5 IBA, root formation will help in acclimatization of resulted bulblets.

**Cytological examination**

Analysis of *Narcissus* cells of native bulbs at mitosis metaphase side view for both genotypes showed that*Narcissus tazetta* var. *Chinensis* have a diploid chromosome number with 30 chromosomes (2n = 30) (figure 5A). While*Narcissus tazetta* cv. Wave also a diploid chromosome number but with only 32 chromosomes (2n = 32) (figure 5B).

Xiao-mei *et al.*, (10), studied the chromosome number for seven cultivars of *Narcissus*sp., and they stated that *Narcissus* classification still indistinct, and the traditional classification has been generally based on morphological features, cytological investigations mutual with morphological analysis allow us to recognize better the classification and the taxonomic relationship between *Narcissus* sp. species. Meanwhile, this examination also delivers the origin to select hybrid parents and classify the hybrid offsprings in these *Narcissus* sp. for upcoming studies and husbandry.

Fig. (5) Cytological examination of *Narcissus*, (A) *N. tazetta* var. Chinensis and (B) *N. tazetta* cv. Wave.




Cytological investigation proposed that analysis could afford the basis for understanding of evolution, geographic distribution and morphology differentiation of *Narcissus*sp. Moreover, analysis had significance to discover the fertility of *Narcissus* sp. hybrid parents, to guide the genetic breeding work, and to clarify the cross combinations between species and the mutation breeding.

**RAPD assay:**

Two groups of the *Narcissus* genotypes (var. Chinensis and cv. Wave) were revealed 26 and 25 bands using the four RAPD primers with polymorphism ratio 50% and 56% respectively. The higher number of bands 9 was shows with primerOPB-07 in Chinensis genotype, while the littlest number of bands5 shows with primers OPA-07 invar. Chinensis genotype and OPA-07 incv. Wave genotype and the molecular weight ranged between (170bp:1300bp) in Chinensis genotype and (70bp:750bp) in cv. Wave genotype (figures 6,7 and tables 5,6).

**RAPD assay for var. Chinensis genotype:**

Chinensis genotype revealed thirty-four bands by using four RAPD primers and the bands molecular weight ranged from 170bp to 1300bp. The first primer OPA-04 showed 6 bands four of them were polymorphic bands by percentage 66.7% and the bands ranged from 500bp to190bp. While primer OPA-07 gave 5 bands two were polymorphic bands by polymorphism 40%. Moreover, the fourth primer OPB-07 revealed the higher number of bands 9 as total bands, four bands were polymorphic bands by polymorphism ratio 44.4% and the bands ranged between 710bp to 205bp.The last primer OPB-10 showed 6 bands half of them were polymorphic bands by ratio 50% polymorphism and the bands ranged from 1300bp to280bp. Table (5) and figure(6).

Fig. (6) DNA banding patterns using RAPD- PCR in var. Chinensis genotype.

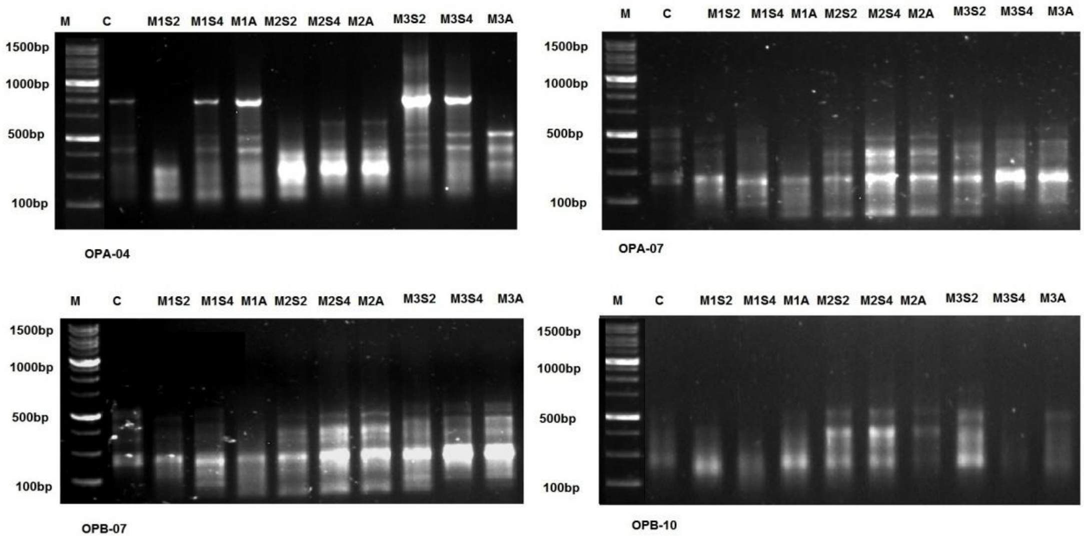



Table (5) Total number, monomorphic, polymorphic of alleles and percentage of polymorphism as revealed using (4) RAPD primers of var. chinensisgenotype

| No. | Primer code | Sequence (5`→3`) | Rang of alleles (bp) | Total bands | Monomorphic bands | Polymorphic bands | % Polymorphism |
|---|---|---|---|---|---|---|---|
| 1 | **OPA-04** | **AATCGGGCTG** | **500-190** | **6** | **2** | **4** | **66.7%** |
| 2 | **OPA-07** | **GAAACGGGTG** | **490-170** | **5** | **3** | **2** | **40%** |
| 3 | **OPB-07** | **GGTGACGCAG** | **710-205** | **9** | **5** | **4** | **44.4%** |
| 4 | **OPB-10** | **CTGCTGGGAC** | **1300-280** | **6** | **3** | **3** | **50%** |
| **Total bands** | | | **-** | **26** | **13** | **13** | **50%** |

**RAPD assay for cv. Wave genotype:**

Data in table (6) and figure (7) of cv. Wave genotype revealed twenty five bands using four RAPD primers and the molecular weight ranged between 70bp to 750bp. First primer OPA-04 showed 6 bands half of them were polymorphic bands, these bands ranged between 750bp-200bp. While primer OPA-07 revealed the heights number of bands 8 bands, five of them were polymorphic bands by 62.5% polymorphism. Moreover, the fourth primer OPB-07revealed six bands, four of them were polymorphic and two monomorphic by polymorphism 66.7% ranged from 510bp-70bp.Finally primer OPB-10 gave 5 different bands, ranged from 510bp-210 with 40% polymorphism.

Fig. (7): DNA banding patterns using RAPD- PCR for cv. Wave genotype.

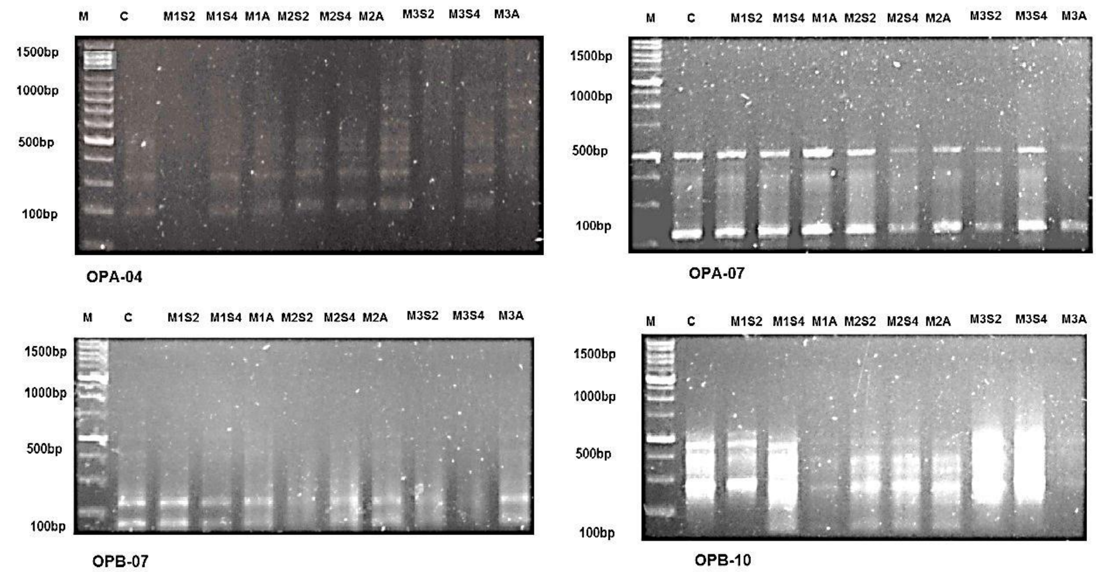



Table 6: Total number, monomorphic, polymorphic of alleles and percentage of polymorphism as revealed using 4 RAPD primers of cv. Wave genotypes.

| No. | Primer code | Sequence (5`→3`) | Rang of alleles (bp) | Total bands | Monomorphic bands | Polymorphic bands | % polymorphism |
|---|---|---|---|---|---|---|---|
| 5 | OPA-04 | AATCGGGCTG | 750-200 | 6 | 3 | 3 | 50% |
| 6 | OPA-07 | GAAACGGGTG | 500-100 | 8 | 3 | 5 | 62.5% |
| 7 | OPB-07 | GGTGACGCAG | 510-70 | 6 | 2 | 4 | 66.7% |
| 8 | OPB-10 | CTGCTGGGAC | 510-210 | 5 | 3 | 2 | 40% |
| Total bands | | | - | 25 | 11 | 14 | (56%) |

RAPD-PCR technique has been used as molecular markers, which is powerful tool in evaluating the genetic relation and diversity, and assessing taxonomic identity of plants [22,54,55,56,57]. The analysis based on RAPD markers showed little genetic variability this is in agreement with previous studies [58,59,60,61]. The results would be revealing RAPD analysis may be more precise than morphological examination, the results would be valuable for phylogenetic analysis, genotype identification besideusing for upcoming breeding studies and practice in the *Narcissus* genus.

In similar studies tissue culture techniques could be induced DNA polymorphisms discovered with RAPD has been reported in some crops as 0.05%, in sugar beet [62] 0.63% in garlic [63] and 3.57%, in soybean [64]. In the other hand some studies had found no RAPD polymorphisms among plants regenerated from tissue culture [65,66,67].

## Conclusion

In the present study a protocol for regeneration (direct and indirect) and subsequent propagation of *in vitro* culture plantlets subsequently, acclimatization was successfully established using different additives to MS medium for two genotypes of *Narcissus tazetta* (*N. t.* var. Chinensis and *N. t.* cv. Wave).

Furthermore, RAPD analysis was used to evaluate the genetic variation of *Narcissus* plantlets regenerated from bulb scales, subculture times and acclimatization of *in vitro* multiplication. Data showed the growth vigor of *Narcissus tazetta.* cv. Wave from bulbs as explants in both direct and indirect micropropagation, as well as medium containing 2mg/L 2,4-D is suitable for callus induction and medium containing 1gm/L of activated charcoal is suitable for direct propagation. Also the two *Narcissus* under the three environments have genetic stability lower than moderate also found that genotypes of *Narcissus* have genetic stability with the change in medium compositions and with different number of subculture plants. The two genotypes had different chromosome number where demonstrated with cytological examination.

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