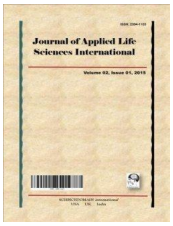

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Micropropagation of *Narcissus tazetta* 'Chinensis' and Its Relation to Secondary Metabolites Content

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Authors' contributions

This work was carried out in collaboration between all authors. Author HMAR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AMFAA and KNR managed the analyses of the study. Author AAR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

In this study, bulbs of *Narcissus tazetta* 'Chinensis' were used as explants for micropropagation. Several disinfection protocols, callus medium and regeneration medium were tested in addition to the secondary metabolites content analysis. Four disinfection protocols were used. The most successful was:1) disinfection of bulbs in 70% ethanol and 2% $HgCl_2$, 10 minutes for each, outside cabinet, or 2) 70% ethyl alcohol (4 min), 20% clorox (5 min), 0.1% mercuric chloride (1 min) and 9% hydrogen peroxide (1 min) inside cabinet, washing with sterilized distilled water after every previous step. This technique is considered as a novel effective procedure. Indirect micropropagation on MS medium supplemented with 2 $mg \cdot L^{-1}$ 2,4-D succeeded in narcissus callus induction which regenerated into shoots on MS medium supplemented with 1 $mg.L^{-1}$ IBA + 1$mg.L^{-1}$ TIBA + 1 $gm.L^{-1}$ of activated charcoal. The same medium with activated charcoal sowed high yield in a direct

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micropropagation protocol. Average shoot and root per explant showed significant increase when using indirect micropropagation. Secondary metabolites from direct and indirect regeneration, using phytochemical screening detected increasing alkaloids content in callus only, while regenerated plantlets were the same as mother plants, as well as there were no significant changes in mother and regenerated plants in terms of total phenol and flavonoids content.

Keywords: Alkaloids; callus induction; daffodils; disinfection protocol; regeneration; Narcissus tazetta.

1. INTRODUCTION

Daffodils (*Narcissus*) belong to the family Amaryllidaceae. They resided in Egypt from Pharaohs era, especially in the northern shores but they almost disappear in the recent years, in spite of all its benefits in terms of plenty of types and forms of flowers and economy of Egypt could be affected, hence it is producing and exporting country from ornamental plants. The plant has also important medicinal values currently used in the extraction of alkaloids and flavonoids. The genus *Narcissus* has been widely used in folk medicine to treat a variety of infections [1]. The plants contain alkaloids which have been isolated and identified for their different biological properties [2]. The amaryllidaceae alkaloids were: narciclasine, galanthamine, haemanthamine, pseudolycorine, lycorine, pretazettin and tazettine [3]. Many therapeutic properties have been established for these alkaloids and flavonoids as: antifungal, antiviral and antitumoral activities [1]. Plant cells as narcissus bulbs have low content of alkaloids, whereas they have a high market price and so made *Narcissus tazetta* L. an essential model plant for biotechnology and secondary metabolism studies[4].

Narcissus tazetta 'Chinensis' is a common flowering ornamentals bulb plant species. It is naturally found in Mediterranean regions [5] with about 60-80 discrete species [6] which cover a wide range of environments. Nowadays, narcissus genus has been focused with expanding attention to their benefits [7]. *Narcissus* development projects are delayed by the slow rate of narcissus vegetative and sexual propagation which yield only 1.6 bulblets annually. It takes nearly about 25–30 years to yield adequate bulbs to complete variety trials and to bulk-up stock for marketable quantity.

Due to low speed of their propagation vegetative methods (chipping and twin scales) is not an effective method. Furthermore, because of a large number of propagation cycles required in the field, conventionally produced bulbs are easily infected [8]. Hence, the tissue culture techniques permit rapid and large-scale spread of uniform plants for field culture [9,10]. Advanced tissue culture procedures could significantly increase the number of narcissus bulbs produced [11]. However, monocots are considered as a challenging *in vitro* material [12]. Many efforts were done to accelerate vegetative propagation by chipping and twin scaling [13], and by *in vitro* micropropagation [14]. *Narcissus* callus culture has been described in a few of their species [15]. Disinfection is an essential preliminary step of a successful micropropagation system which requires cautious optimization [16].

The aim of this study is to establish a new effective disinfection protocol and rapid multiplication tissue culture system through direct and indirect regeneration from different parts of bulbs and leaves of *Narcissus tazetta* var. Chinensis. This experiment was planned to study the effect of culture media composition on bulblets induction, multiplication and related secondary metabolites content.

2. MATERIALS AND METHODS

2.1 Plant Material

Two years old bulbs of *Narcissus tazetta* 'Chinensis' bulbs were collected from a local producer.

2.2 Disinfection of Bulbs and Explants

Firstly, healthy two-years-old narcissus bulbs were carefully cleaned under running tap water, then outer scales and roots were removed with keeping basal plate intact. The cleaned bulbs were soaked in distilled water with few drops of soap and clorox, and kept for half an hour, next bulbs were washed for 1 hour by running tap water, then disinfection procedure was started as follows in Table 1.

Table 1. Different disinfection procedures for narcissus explants

Disinfection protocols		
Protocol No.	**Outside cabinet**	**Inside cabinet (Washing with sterilized distilled water was done after every step)**
1st	1- Soap + clorox in water for 30 min 2- Washing by running water for 1 h 3- Spray the bulbs with 70% ethanol	1-Bulbs were submerged in 70% ethanol +2-3 drops of Tween 20 for 1 min followed by washing twice. 2-20% Clorox + 2-3 drops of Tween 20 for 5 min followed by washing twice.
2nd	The same steps as 1st protocol	1-Bulbs were submerged in 70% ethanol + few drops of Tween 20 for 1 min followed by washing twice. 2- 20% Clorox + few drops of Tween 20 for 5 min followed by washing twice. 3- Bulbs were kept in warm water for 1 h 4- 20% Clorox + Tween 20 for 5 min again followed by washing twice.
3rd	The same steps as 1st protocol	1-Bulbs were submerged in 70% ethanol + few drops of Tween 20 for 1 min followed by washing twice. 2- 20% Clorox + few drops of Tween 20 for 5 min followed by washing twice. 3- 0.1% Mercuric Chloride for 1 min followed by washing three times. 4- 9%$H_2O_2$for 1 min followed by washing once.
4th	1- Cleaned bulbs soaked in water with soap + clorox for 30 min 2- Washing with running tap water for 1 h 3- Soaking bulbs in 70% ethanol for 10 min 4-Soaking bulbs in 2% $HgCl_2$ for 10 min 5- Spray the bulbs with 70% ethanol	1-Bulbs were submerged in 70% ethanol + few drops of Tween 20 for 1 min followed by washing once. 2- 20% Clorox + few drops of Tween 20 for 5 min followed by washing twice. 3- 0.1% Mercuric Chloride for 1 min followed by washing three times.4- 9% H_2O_2 for 1 min followed by washing once.

2.3 Culture Media

Culturing medium was MS [17], supplemented with different plant growth regulators (PGRs) such as indole-3-butyeric acid (IBA), 2,4-Dichloro-phenoxyacetic acid (2,4-D) and 2, 3, 5 triiodobenzoic acid (TIBA) at a single concentrations of one or more addition depending on the goal as following:

2.3.1 Indirect propagation

MS medium supplemented with 2 $mg.L^{-1}$ 2,4-D + 30 $g.L^{-1}$ sucrose + 7 $g.L^{-1}$ agar (pH 5.8).

2.3.2 Direct propagation

MS medium supplemented with 1 $mg.L^{-1}$ IBA + 1 $mg.L^{-1}$ TIBA + 30 $g.L^{-1}$ sucrose + 7 $g.L^{-1}$ agar in addition to 1 $g.L^{-1}$ of activated charcoal (AC), (pH 5.8).

2.4 Culture Establishment

- Leaf bases were cut 10 mm above the basal plate and were left attached to a 1.5 mm length of basal plate.
- Bulb explants were divided longitudinally into three or four parts depending on the bulbs size (Twin-scales). Green leaves were used as other explants for culture initiation.
- Explants were cultured on callus induction medium (2 $mg{\cdot}l^{-1}$ 2,4-D) incubated in the dark conditions at 18 ± 2°C and were observed weekly, then after 3-5 weeks, jars with calli were transferred to light followed by transferring to standard regeneration medium (MS with activated charcoal 1 $gm{\cdot}l^{-1}$) after 2 weeks.
- One subculture was done after 4 weeks of regeneration culture initiation, cultures were incubated at 18 ± 2°C, for 16/8 h

day/night photoperiod (one explant was used in each jar with seven replicates).

2.5 Phytochemical Analysis of the Samples

Air-dried plant sample (1 gram) was extracted with 5ml methanol at 50ºC. Methanol extracts were concentrated under vacuum, then 1 ml were taken from each plant extract samples. Four sample types were used as following: (1) mother plant, (2) callus, (3) regenerated plant and (4) subcultured bulblet (7 replications plant from each combination), followed by dissolving in methanol solvent and few drops of distilled water was added for complete solubility and then the extract was subjected to different phytochemical tests described by Yadav and Agarwala [18].

2.6 Determination of Total Phenolic and Flavonoids Contents

The total phenolic contents were analyzed by the colorimetric method [19], with minor modifications, 0.5 mL of sample (1 mg) was mixed with distilled water (7.0 mL) and subsequently with Folin-Ciocalteu's phenol reagent (FC reagent) (0.5 mL). After sixmin, Na_2CO_3 solution (2.0 mL, 2.0%) was added into the mixture. The observed color absorbance was measured by a spectrophotometer at 760 nm after 90 min. The total phenolic contents were expressed as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph (0-400 µg mLG1) (R^2 = 0.9998).

The total flavonoids contents were determined by using a modified colorimetric method described previously by Dewanto et al. [20]. The 1.0 mL of the extract was added to test tubes containing DMSO (5.0 mL) and subsequently $NaNO_2$ solution (1.0 mL, 5.0%) was added to each of them. After 6 min, $AlCl_3$ solution (1.0 mL, 10%) was added to the tubes and the mixtures were allowed to stand for 5 min before further addition of NaOH solution (2.0 mL, 1.0 M). The absorbance values were read at 510 nm. Total flavonoids contents were expressed as microgram of catechin equivalent that was obtained from standard graph (0-200 µg mLG1) (R^2 = 0.9977).

2.7 Statistical Analysis

Results are expressed as the mean ± standard deviation (S.D.) of three independent experiments, t-test was used for statistical analyses; P values > 0.05 were considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Disinfection Procedures

In this research four procedures were used as previously mentioned (Table 1). The 4th procedure was the most successful one (Table 2). The 1st procedure had the highest bacterial and fungal contamination, the 2nd procedure had moderate bacterial and high fungal contamination, while the 3rd procedure showed (16%) contamination. Moreover, the percentage of the 4th protocol was 6% and this could be attributed to using mercuric chloride. This is in agreement with Jevcsak [21]. In comparison, Lema-Rumińska and Kulus [22] and Kulus [23] obtained an over 80% disinfection efficiency of *Astrophytu masterias* and *Kalanchoe tubiflora* primary explants by applying sodium hypochlorite.

The effectiveness ofthe 4th protocol to eliminate bacterial and fungal contamination, could be attributed to the use of 2% mercuric chloride and 70% ethanol followed by hydrogen peroxide (9%) and mercuric chloride (0.1%). Whereas, using warm water in the second protocol may enhance the fungal spores to grow, while the first protocol was not enough to eliminate any contamination.

3.2 Callus Induction

In this study, two types of media with different components were used. Results showed that MS

Table 2. Results of the disinfection protocols for *Narcissus tazetta* bulbs

Disinfection protocol	Type of contamination	Contamination percentage of cultured jars
1st	Bacterial and fungal contamination	100%
2nd	Bacterial and fungal contamination	55%
3rd	Bacterial and fungal contamination	16%
4th	Bacterial and fungal contamination	6%

+ 2,4-D medium was suitable for callus induction. Indirect micropropagation protocol was give 70% of bulb propagation frequency and growth, whereas MS medium supplemented with active charcoal gave the best results for callus regeneration. Callus mostly was yellowish-white color and compact, as shown in (Table 3).

It was clear that main bulb was the best part to be used as explant. The results showed that twin scale method of the main bulbs recorded (70%)of calli induction (table 3) while leaves never gave any callus (Fig. 1). It is concluded that media components used in this study can be recommended for callus induction and regeneration. This is in agreement with Li et al. [7], Zaidi et al. [12], Jevcsak [21], Park et al. [24] and Sage et al. [25].

The plant growth regulators used in this study were auxins as following, IBA,2,4-D,IAA and TIBA. Auxins stimulate numerous cytological activities, such as celldivision and elongation, vascular tissue proliferation and root formation [26]. Indirect propagation gave a significant increase in shoots number (6.5±2.1*) and roots length (7.2±1.5*) compared to direct organogenesis, as illustrated in Table 3. In the contrary, shoots length in direct method was significantly greater (3.2±1.8*) when compared with indirect method (Table 3).

3.2 Regeneration

The obtained calli gave regenerated plantlets (90%) after they were sub-cultured on MS medium with activated charcoal. Fig. 1 shows the callus induction stages, figure (2) shows regenerated plantlets and roots. Narcissus production encountered by many conflicts infections as viruses and pests besides the low rate conventional propagation [25], therefore, crop improvement hindered with slow reproduction [26], it takes about 25–30 years to produce enough bulbs for commercial release [27]. This study shows that daffodil commercial growth could be accelerate using tissue culture techniques and to overcome many encountered problems, as the slow rate of normal vegetation production.

Callus induction in *Narcissus* genus has been described only in a limited number of species [8]. Similar to other monocots, the species are considered a difficult *in vitro* material [9], but the use of sophisticated tissue culture techniques, can increase the number of produced plants [13]. Santos et al. [28] studied the culture conditions for *in vitro* propagation of *Narcissus asturiensis* bulbs. Twin-scales were used as primary explants, then cultured on a modified MS medium supplemented with IBA (1 $mg.L^{-1}$), BA (1.99 $mg.L^{-1}$) and NAA (0.12 $mg.L^{-1}$), BA (5.99 $mg.L^{-1}$). Both media were found suitable for shoot initiation and proliferation, but the growth rate of leafy shoots was higher with NAA and BA. Small bulb appeared at the leave bases after 60 days. Jiao et al. [8] suggested that *in vitro* anther culture can give a novel indirect micropropagation method in Chinese narcissus. Whereas, Sage [29] established a novel technique for fast and effective propagation using bioreactors for *Narcissus pseudonarcissus*.

Fig. 1. Callus induction of the *Narcissus tazetta* indirect micro propagation: a) from green leaf and b) from leaf base

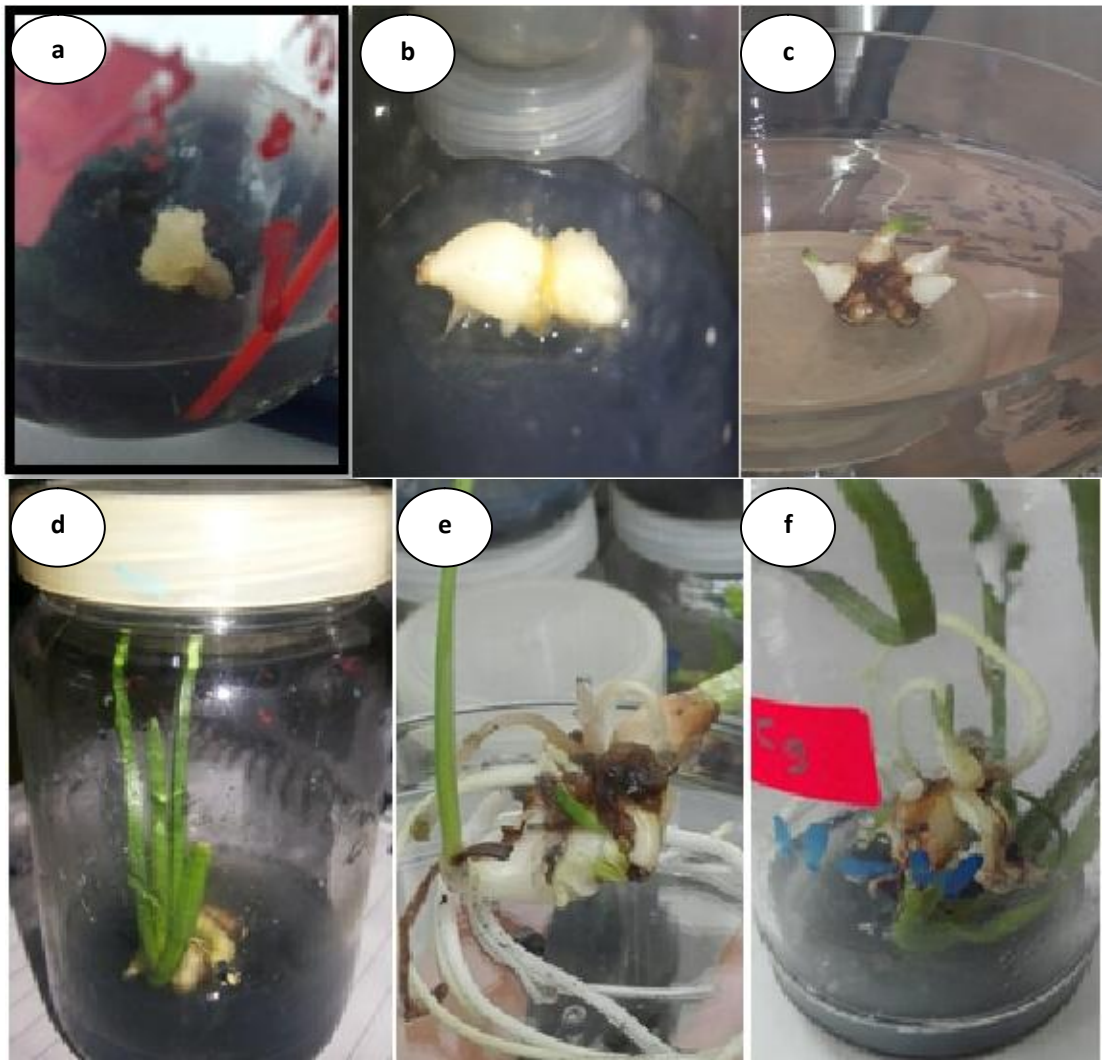

Fig. 2. Different stages of the *Narcissus tazetta* micropropagation: a) Callus induction on 2,4-D-supplemented media b), Embryo formation, c) Starting of regeneration, d) Regenerated plantlets, e) Root formation of the regenerated plants and f) Bulblets from twin scale method of leaf base

The results of this study demonstrated that twin-scale explants with basal plate were more suitable as an explant as mentioned in (Table 3 and Fig. 1). Medium supplemented with 2 $mg.L^{-1}$ 2,4-D was appropriate for callus induction, as well as MS media supplemented with 1 $mg.L^{-1}$ IBA + 1 $gm.L^{-1}$ TIBA and activated charcoal found to give excellent results. Activated charcoal (AC) is frequently used in plant tissue culture to increase the growth and development of plant cell, hence AC has a very fine net of holes, this means it has a large area in their inner surface to enable numerous elements to be adsorbed [30]. The addition of activated charcoal to media enhances and affects the growth and development of explant and regenerated plant, and this may be attributed mostly to the adsorption of growth-inhibitors present in the culture medium [31]. Due to AC presence, the phenolic oxidation was extremely reduced and brown excretions accumulation eliminated [32], medium pH was altered to an optimum degree suitable for morphogenesis [33]. These offering conditions by AC addition stimulate root growth and hence, plantlet growth increase.

Table 3. Micropropagation of *Narcissus tazetta* with direct and indirect procedures. (AC= Activated Charcoal). (*= significance at 0.05, T test)

Protocol	Number of explants (twin scale method)	% of Callus induction	Incubation conditions	Callus properties	Average shoot per explant		Average root per explant	
					Number	Length [cm]	Number	Length [cm]
Indirect	120	70%	Jars with explants kept in dark for 3-5 weeks then jars transfer to light followed by subculture in MS with AC for 4 weeks	Yellowish white color and compact	6.5±2.1*	2.6±0.7	3.9±0.3	7.2±1.5*
Direct	120	10%	Jars with explants kept in light for 4 weeks then subculture to MS with AC for 4 weeks	Brownish yellow color and compact	4.6±1.6	3.2±1.8*	4.7±0.5	6.2±0.9

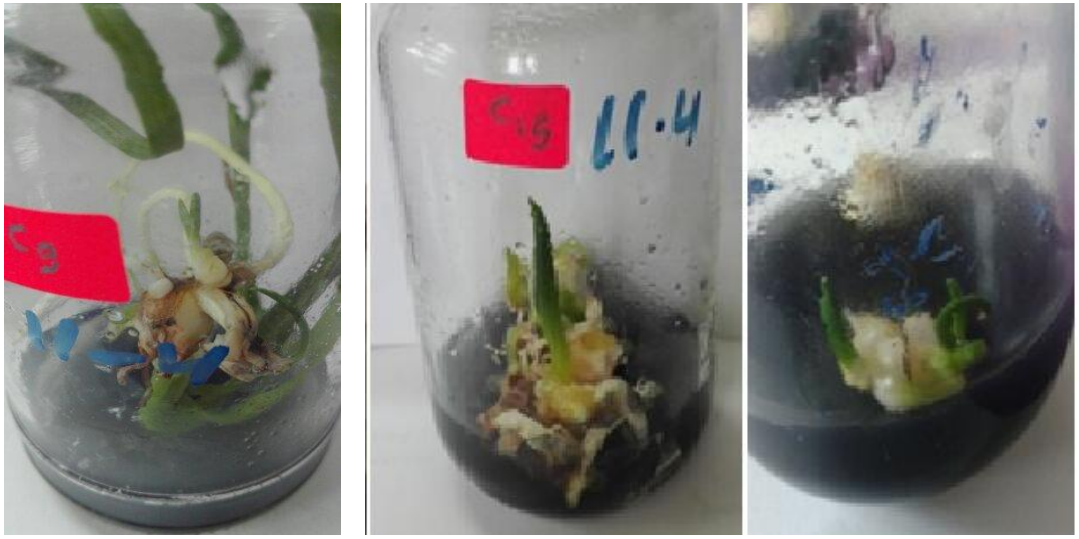

Fig. 3. Direct micropropagation of *Narcissus tazetta* on MS medium with activated charcoal

In several bulb plants, induction of bulblets is significantly affected by the presence of activated charcoal in the medium [34,35]. Activated charcoal increases growth and development of plant cell when used in tissue culture procedure. It shows a dynamic role in micropropagation enhancement response and the obvious increase in bulblet size [34]. In addition, Steinitz and Yahel [35] reported that AC stimulated bulblet production in *Narcissus tazetta*, which is in agreement with our results.

3.3 Phytochemical Screening

Results of constituents for all micropropagation stages compared to original plant showed in table (4) exhibited no difference between the original mother plants and other plant samples that regenerated in both MS media. While total phenolic and flavonoids were listed in table (5), data showed that there were no significant changes between different micropropagation stages, calli and mother plants in their contents of total phenolic and flavonoids content.

Results proved that all samples contained the same quantities of secondary metabolites, except one changein alkaloids content found only during callus induction. Other samples, such as: mother plants, regenerated or subcultured plantlets were found to have the same amount of carbohydrates, glycosides, alkaloids, sterols, triterpenes, and coumarins.

Amaryllidaceae family plants are well-known not only for their ornamental importance, but also they are remarkable for their alkaloids contents [4]. Many methods have been planned to increase the alkaloid manufacture under tissue culture conditions, for example via optimization of media conditions [36,37]. Different combinations of growth regulators (auxins and cytokinins) which will be added to micropropagation media, are capable of generating the same types and quantity from secondary metabolites as resulted from the integral plant while, higher levels of secondary metabolites will be resulted with media conditions optimization [4]. Furthermore, techniques of plant tissue culture provide another possibility to produce secondary metabolites that have marketable importance in food and medicine products [38]. In addition, micropropagation provide several benefits above the *in vivo* agriculture ofentire plant since the removal of microbial contamination and the absence of the environmental elements (as light, nutrients and temperature) [39].

Table 4. Phytochemical screening of *Narcissus tazetta* donor plants, callus and regenerated plantlets produced on MS media with activated charcoal (AC) and subcultured bulb. (+) denotes the presence of the constituent, (++) denotes the excess presence of the constituent and (-) denotes the absence of the constituent.

Constituent	Biological material			
	Original bulb	Callus	Regenerated plant	Once subcultured bulb
Carbohydrates and/or glycosides	+	+	+	+
Tannins	-	-	-	-
Alkaloids	+	++	+	+
Flavonoids	+	+	+	+
Sterols and/or Triterpenes	+	+	+	+
Saponins	-	-	-	-
Coumarins	+	+	+	+

Table 5. Quantity of methanol extract for total phenolic and flavonoids contents in different micropropagation stages of *Narcissus tazetta* mother, callus and regenerated plantlets on MS media with activated charcoal (AC) and subcultured bulb. (*= significance at 0.05, T test)

Constituent	Biological material			
	Mother bulb	Callus	Regenerated plant	Once subcultured bulb
Flavonoids	13.86±0.01	13.89±0.02	13.62±0.022	13.60±0.022
Total phenolic	143.29±0.01	144.06±0.01	143.11±0.019	143.05±0.018

In another approach, Singh [40], found that both biotic and abiotic stresses lead to enhanced secondary metabolites production in plant tissue cultures. In several studies, various parameters, such as growth regulators and nutrients in the medium, could affect the secondary metabolites production [41]. In addition, Verma et al. [42], described an increase in total alkaloid content of calli induced in MS media supplemented with 1:0.5 and 0.5:1 mg/l of 2,4-D and BA, while Kadi et al. [43] and Abu Taleb et al. [4], studies showed that alkaloid production in *Narcissus tazetta* 'Italicus' is improved by cytokinins and auxins interaction. These are in agreement with our studyin case of callus stage.

4. CONCLUSION

This research proved that *Narcissus tazetta* 'Chinensis' bulbs could be directly or indirectly micropropagated with fewer contamination percentage and maximum results of callus induction and regeneration. First of all, with applying a new an effective disinfection procedure outside and inside the culture cabinet, consequently, by using 2,4-D-supplemented medium indirect callus induction results was 70%. After that, the induced calli were regenerate indirectly and directly to overcome its slow vegetative reproduction using MS medium supplemented with activated charcoal. The quantity of secondary metabolites constituents extracted from the regenerated plantlets have no change under all procedures and protocols that were used in this study, but with one exception in case of callus but with little non significance increase. Total phenolic and flavonoids compounds were recorded non-significant changes after micropropagation. This study could contribute to enrich and increase the diversity of narcissus in the desired direction.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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