Middle East Journal of Applied Sciences **Volume : 10 | Issue :03 |July-Sept.| 2020**
EISSN: 2706 -7947 ISSN: 2077- 4613 **Pages: 564-571**
DOI: 10.36632/mejas/2020.10.3.48

Establishment of tissue culture study for *in vitro* propagation of *Fortunella margarita* from callus induction for shoot regeneration

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Received: 15 August 2020/ Accepted 10 Sept. 2020 / Publication date: 20 Sept. 2020

ABSTRACT
This work deals an establishment for *in vitro* propagation of Kumquats (*Fortunella margarita*.) from callus for shoot regeneration**.** Two types of explants (cotyledons and shoot tips) and two kinds of cytokines (BA and TDZ) at various concentricity from 0.1 to 4.0 mg/L, plus basal medium without any growth regulators as a control were used. Addition of the both of cytokines at levels 0.1 mg/L to 4.0 mg/L to WPM medium to both shoot tip and cotyledon explants gave 100 % of shoots proliferation with non-formation of callus. Shoot tip explants at 4.0 mg/L from both of two cytokine's produced shoots with callus formation. The cotyledons explants at 0.1 to 2.0 mg/L for both of two cytokines produced shoots and callus together. All the concentrations of both cytokines from 0.1 mg/L to 4.0 mg/L did not produced any roots. The media without any cytokines at 0.0 concentrations prevented development of callus, shoot, and root production. The cotyledons callus morphology was different than that derived from shoot tip. The shoot tip explants produced a yellow callus fail to format to shoots directly, and the yellow callus turn to brown callus when it transferred to the embryo induction medium, while the cotyledons explants produced a compact green embryonic callus when it transferred to direct shoot proliferation media. The highest concentrate (4.0 mg/L) from both cytokines produces the highest shoot number.

***Keywords*:** *Fortunella margarita*, tissue culture, TDZ, BA, WPM, plant production.

Introduction

Kumquats (*Fortunella* spp.), an evergreen small shrubs of *Rutaceae*, related to *Citrus* genus plants. Kumquats ripe fruits bit similar to tomatoes cherry in shape and size; the color of the ripe fruits ranged from yellow orange to red orange. The fruits of kumquats rich in vitamins (A and C), fiber, and contains traces of iron and calcium. Techniques of *in vitro* propagation made the improving against different abiotic stresses is easy, conserving for citrus genotypes by utilization soma clonal variations and somatic cell hybridization became easy (Deng *et al*., 2000).

The embryo genetic calli obtained from Citrus genotypes cannot differentiate to somatic embryos through losing their ability to somatic embryogenesis. Embryogenesis obtained easily from immature or premature embryos of Citrus species callus (Zhang *et al*., 2006), the success of embryo culture achieved at less of time with the methods of conventional breeding with non-effective result. Plant growth regulators and physical are an important factor for the embryonic growth and differentiation of kumquats (Yang *et al*., 2006) mentioned that *Fortunella crassifolia* embryonic callus was obtained on MT medium with 2% glycerol. The somatic embryos from leaves of 'Valencia' produced Non-embryogenic callus (NEC) in MT medium with 1.5 mg/L 2,4-D (Hatice and Dereboylu, 2016) reported that growth regulators have an important effect to develop a specific growth in the tissues or cells cultured, according to the accumulation of biochemical cells contents. The one type of growth regulators or a combination between different types from growth regulators in a media causes organic and inorganic specific balance content for tissue growing. That makes the tissues or cells developed into shoots/or roots or even death.

The acclimatization for the developing plants *in vitro* is the most critical step in the establishment yield for in vitro propagated plants. The achievements of plant growth and high survival rate demand a very good greenhouse condition, and a modification of internal microclimate to create a local environment (Mondal *et al*., 2004; Vijayan *et al*., 2011).

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This study were planned to identify the ideal method for the micro propagation of Kumquats *Fortunella margarita* and try to establish an sufficient regeneration plantlet *in vitro* by using the shoot tips and cotyledon explants of kumquat.

Material and Methods

This work was done in the tissue culture lab at applied research center of medicinal plants and natural products during the period from 2011 - 2018.

1. Plant material and preparation of explants:

The seed was collected from the fruit of (kumquat) *Fortunella margarita* thin subjected to the surface sterilization by 70 % ethanol for 1 min, followed by 0.2 % mercuric chloride for 5 min, and rinses for 3 times with sterile distilled water. After sterilization the seeds were sown into the propagated trays contained mixture of peat moss and vermiculate at 1:1 (v/v). Germination procedures was made in the greenhouse conditions. After 25 days, the kumquat seedlings were transferred from trays to tissue culture lab and the seedlings roots were removed then washed thoroughly three times with tap water, after that they sterilized by dipping in 1% sodium hypochlorite for 5 min and rinsed five times in sterilized distilled water. The culturing for both shoot tips and cotyledons explants was done inside the laminar flow hood. Shoot tips explants cut under the microscope to 0.3-0.5 mm in length, while the cotyledon explants cut into 1.0 cm.

2. Nutrient media:

The basal nutrient woody plant medium (WPM) (Lloyd and McCown, 1980) contained macro and microelements was used during the establishment, and shoot proliferation stage. While, macro and microelements of (MS) (Murashige and Skoog medium, 1962*)* medium was used during the rooting stage. PH of the media was adjusted at 5.7 + 0.1 before the addition of 7 g/L from agar. The media was distributed into tube each jar contained 30 ml of the sterilized medium and autoclaving at 121˚C for 15 min.

3. Treatments:

For shoot regeneration, both of the shoot tip and the cotyledon explants were transferred to WPM basal nutrient medium supplemented with thidiazuron (TDZ) and benzyl adenine (BA) at concentrations of 0.0 (control), 0.1, 1.0, 2.0 and 4.0 mg/L for each. Average number and length of proliferated shoots was recoded after 4 weeks. The morphogenesis responses of shoot root and callus formation were observed.

4. Somatic embryogensis

The produced calluses from all previous treatments was transferred to the MS medium as an embryo induction medium plus 2 mg/L 2,4-D + 0.5 mg/L Kin + 2 mg/L NAA according to Hatice and Dereboylu, (2016) for embryonic callus (EC).

5. Rooting and acclamatization:

For rooting development, all the shoots (without any callus) obtained were cultured on the rooting medium plus IBA at 2.0 mg/L. for 30 days, all complete plantlets transferr to plastic pots filled with peat-moss and vermiculate at ratio of 1:1 (v/v) and puted in the green house under mist for adaptation. The plantlets was fertiliz by Hoglands solution once a week for period of 45 days.

6. Culture conditions:

The culture tube were kept under the constant temperature 20-26˚C + 2 with fluorescent light of 1500 Lux for 16 hours photoperiod. The morphogenetic characters percentage of, shoot number/explants, shoot length and callus, for both shoot tip and cotyledons explants were recorded after 30 days from culture.

7. Experimental design

The experiments were subjected to the completely randomized design with four replicates each replicate was resembled by 10 explants. Analysis of variance (ANOVA) and "LSD test**,** was performed to analyze the obtained data (Snedecor and Cochran, 1982). The difference among averages of all recorded parameters was tested for significance at 5% level.

Results and Discussion

1. Morphogensis:

Both of shoot tips and cotyledons explant which cultured in the WPM basal medium without any cytokinins (control) failed to give any response as shown in Table (1).

Table 1: The effect of cytokinins type and concentration on the morphogenetic characters of shoot tips and cotyledons explants.

Tratments (mg/L)	Shoot formation		Root formation		Callus formation	
Explant	Shoot tip	Cotyledon	Shoot tip	Cotyledon	Shoot tip	Cotyledon
Control	-	-	-	-	-	-
TDZ						
0.1	+	+	-	-	-	+
1.0	+	+	-	-	-	+
2.0	+	+	-	-	-	+
4.0	+	+	-	-	+	-
BA						
0.1	+	+	-	-	-	+
1.0	+	+	-	-	-	+
2.0	+	+	-	-	-	+
4.0	+	+	-	-	+	-

(+) formed , **(-)** unformed

The plant cell and organ cultures successes depended on the used media. Adding of both cytokinins from 0.1 to 2.0 and 4.0 mg/L in the media lead to both of shoot tip and cotyledon culturing gives 100% direct shoots formation. The formmation of shoots and callus together of the shoot tip explan were obtained when its cultured on a media supplemented with 4.0 mg/L from both TDZ and BA. While the cotyledons explant formed shoots and callus in a media containing each of cytokinins at 0.1 to 2.0 mg/L. Our result are in agreement with Thirunavoukkaras *et al*., (2010), they mentioned that the high concentration from cytokinins induce both auxiliary and adventitious shoot formation from meristematic explants and reduce the apical meristem dominance. A higher concentration from BA inhibits the adventitious meristems elongation and inhibits the conversion to a complete plants in banana as reported by Khalid, (2011).

Data in Fig (1) observed that the number of shoots per explants depending on the the type of explants, the type of the cytokinins and the concentrations. Shoot tip explants was the best for forming more number of shoots than the cotyledon explants. (Fig 1:A). Data also cleared that TDZ at a high concentrations significantly activated the formation of shoots than BA (Fig 1: B).

That results are coincided with El-Zeiny, (2007) who reported that TDZ was better than BA for increasing number of shoots of *Cucumis sativus* L. Fig (1: C) clears that increasing of the concntration from 0.1 to 4.0 mg/L was enough to produce more shoots especially 4.0 mg/L gave highest shoot number. That result in agreement with Yang *et al*., (2006), they illustrated that there was a direct relationship between high concentrations from the cytokinins and increasing in number of shoots because the cytokinins presence in a medium depressed apical dominance and consequently activate axillary buds, that leads to increasing proliferation. Raising the cytokinins concentrations increase the proliferation of buds and formation of multi apexes plantlets. An interaction between treatments as presented in (Fig 1: D), lead to that shoot tip or cotyledons explants cultured into medium containing TDZ at 4.0 mg /L enough to produced highest number of shoots (7 shoots) for shoot tip explant and (6 shoots) for cotyledons explant, respectively when its compare with other treatments.

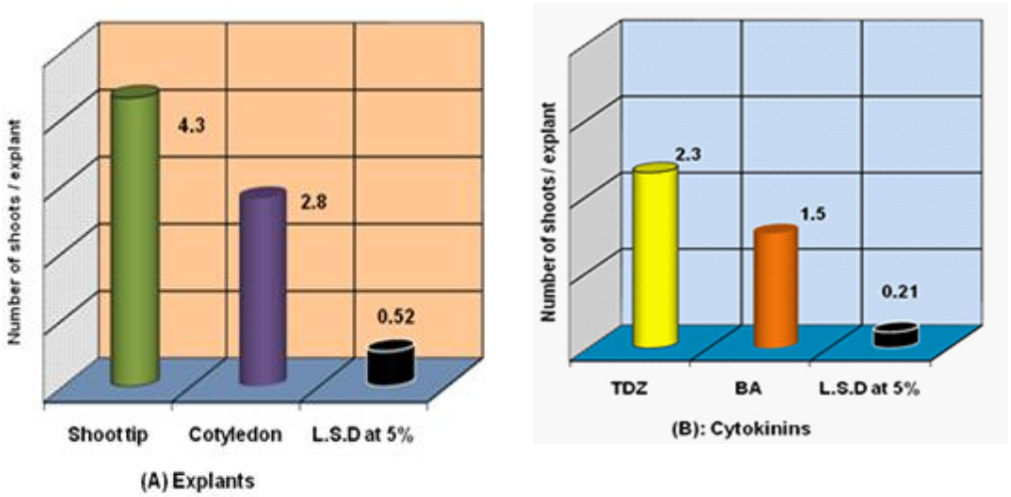

Fig. 1: A and B: Effect of the type of explants (A) and the type of cytokinins (B) on the shoot number per explant of Kumquats .

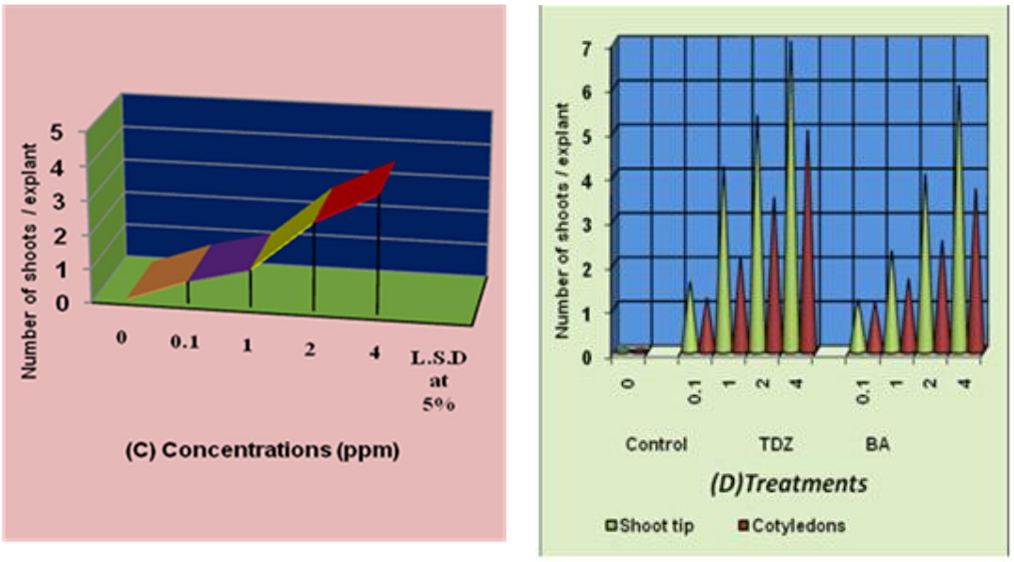

Fig. 1: C and D: Effect of cytokinins concentrations (C) and the interaction between treatments {(control, TDZ and BA) ,(D)} on the number of shoots per explants of Kumquats

Data in Fig (2: A.B.C and D) illustreated that shoot tips explant and BA were most suitable kind to increase shoot length (Fig 2: A and B). Data also cleared that increasing the cytokinins concentration at 0.1 to 4.0 mg/L reduce the shoot length. (Fig 2: C), and the shortest shoots was achevied on the highest rate of both cytokinins at 4.0 mg/L because of the high number from the obtained shoots on the highest level of (4.0 mg/L) which slow down the uptake from the nutrient medium and causing less in shoot length (El-Zeiny, 2007). cleared that the addition of BA into culturing media for shoot tip explants of *Cucumis sativus* L. significantly produce the longest shoots than the TDZ applications (Fig 2: D).

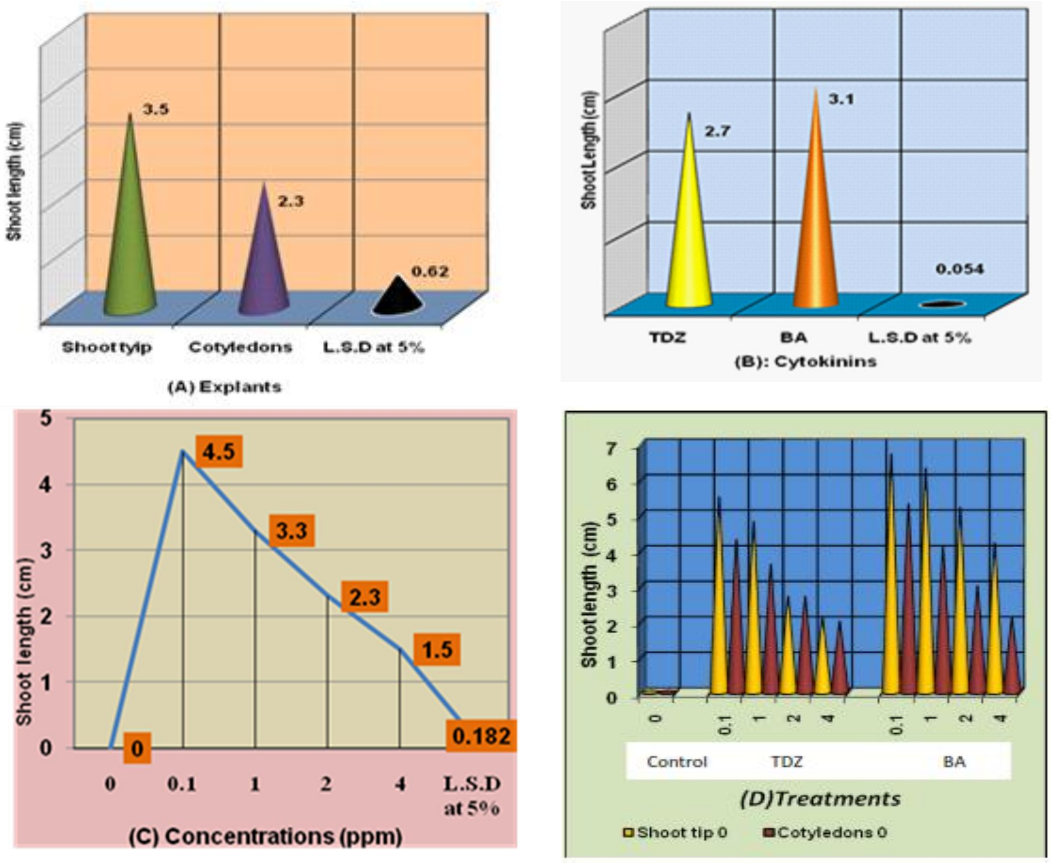

Fig. 2: A, B , C and D: Effect of explants typ (A) , cytokinins kind (B), the concentrations (C) and the interaction between treatments (D) on the shoot length of Kumquats.

2. Somatic embryogensis:

The produced callus morphology of cotyledon was diffed than that drived from shoot tip. Cotyledons explant produce green embryonic compact callus tissue as shown in (Fig 3), which directely gave shoot proliferation after transferring to the embryo induction medium as shown in (Fig 4). While, shoot tip produce a yellow callus tissue as shown in (Fig 5) and fail to regenerate to direct shoots when it transferred into the embryo induction medium. These result are in agreament with Hasan *et al*. (2016) when they obtained callus from Citrus (*Japonica Margarita*). The differences in the ability to form a somatic ebryogenesis due to physiological development differences or to the varying response to the same plant growth regulators. That result are is agreement with Hatice and Dereboylu, (2016) they found that the embryogenesis easily formed from the callus of premature or immature embryos of Citrus species of the *Fortunella japonica* (Thunb) cotyledone explants .

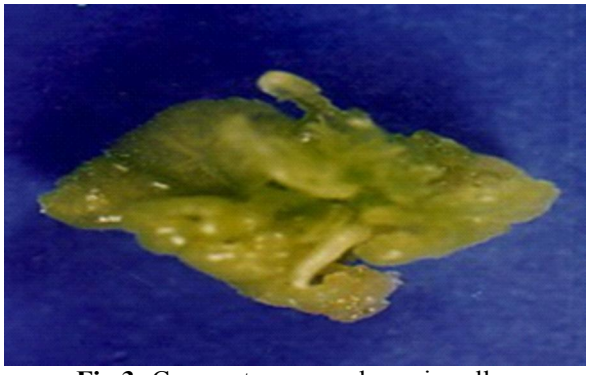

Fig 3: Compact green embryonic callus

Fig. 4: Shoot proliferation of the embryonic cotyledon from callus cultured on the induction medium.

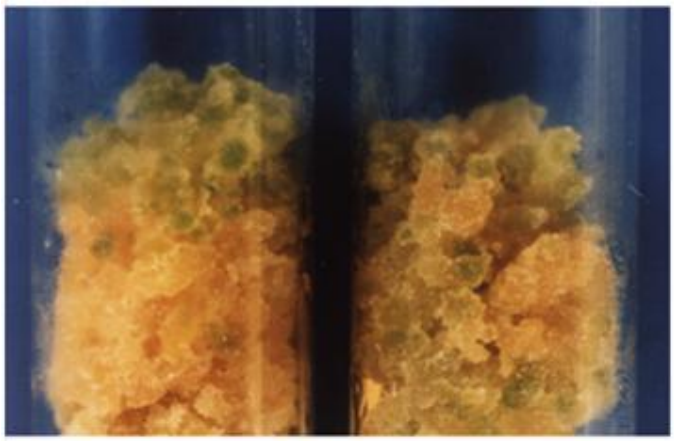

Fig. 5: Yellow callus tissue

Shoot regeneration of cotyledons callus culture as in Fig (6) showed that, increasing concentrations from 0.1 to 4.0 mg/L led to have a significant increasing in the percentag of embryonic callus and number of regenerated shoots. The maximum number of shoots were obtained at 4.0 mg/L in both cytokinins (TDZ or BA) . Data illusterated that TDZ is significantly superior than BA for giving highest embryonic callus percentage or much more number of regenerated shoots as presented in Fig (6).

3. Rooting and acclamatization:

All obtained shoots were transferred to rooting medium contain IBA at 1.0 mg/L. After 4 weeks the complete produced plantlets were transferred to pots filled with peat-moss and sand at ratio of 1:1 (v/v) into the greenhouse under mist condetion. All plantlets were fertilized with Hoglands solution once a week for a period of 45 days with 85% survival percentage. All the survival plants were transplanted into the field to obtain a trees of Kumquat. After 5-7 the trees produced fruits which the essitional oil extecracted from it has a medicinal penefites.

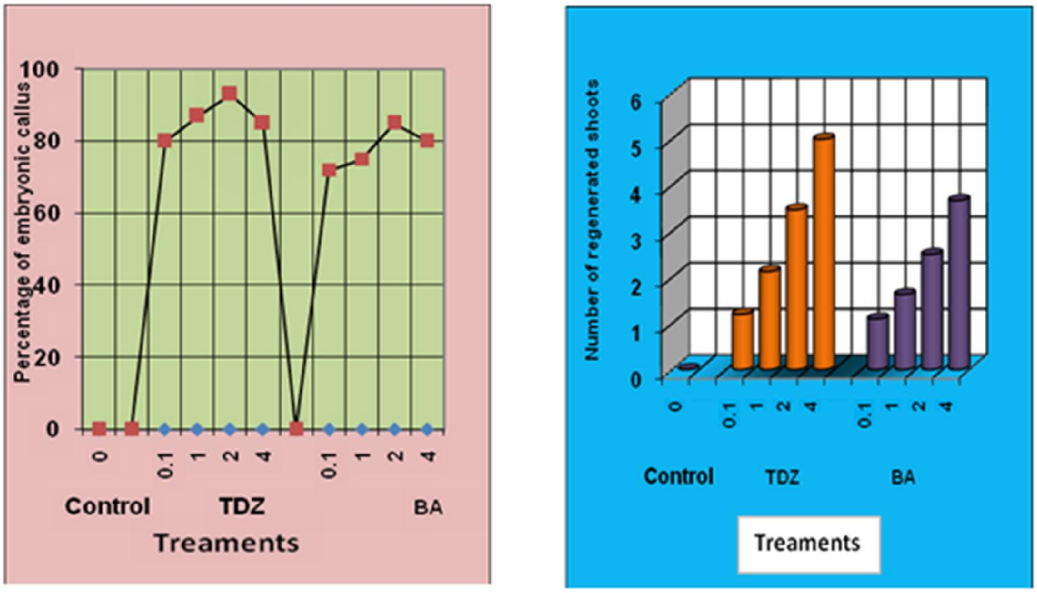

Fig. 6: Percentage and number of shoots regeneration of Kumquat developed from callus culture of cotyledons explants after transferring to the embryo induction medium.

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