Studies on Initial Shoot Tip Culture of Oval Kumquat (*Fortunella margarita* Swingle) *in Vitro*

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Key words: Plant growth regulators, Kumquat, Tissue culture

Summary

The objective of this study was to develop an efficient method for propagation of oval kumquat by tissue culture. Shoot tips about 1 cm in length were harvested in spring, shoot apices in 0.5 mm length were excised under microscope and cultured on MS solid medium containing 30 g/l sucrose, 8 g/l agar for the highest survival percentage in initial culture. Survived explants appeared good growth after transferred to MS solid medium containing 30 g/l sucrose, 8 g/l agar adding 0.1 mg/l BA, 0.01 mg/l IBA and 0.1 mg/l kinetin.

Introduction

Kumquats (*Fortunella* spp.), also called kinkan, which is small evergreen shrubs of *Rutaceae*, closely related to plants of *Citrus* genus. Kumquats are commercially cultivated in China, Japan, United States, *etc* and routinely propagated by grafting, however, strictly technical and seasonal requirements largely handicapped its application. There have been studies on shoot tip culture of evergreen fruit tree, wax apple (Yu and Yang, 1989), passionfruit (Yu *et al*., 1993), guava (Lee and Yang, 1994), atemoya and cherimoya (TsaiHuang *et al*.,

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1998), loquat (Wang and Yang, 1999), Indian jujube (Yeh and Yang, 1999), Ceylon olive (Lo and Yang, 2000; Lo and Yang, 2006), carambola (Lin and Yang, 2002), avocado (Liao and Yang, 2005), mango (Yang and Ludders, 1993; Thomas and Ravindra, 1997), longan (Wang, 2001). For kumquats, however, studies on shoot tip culture are quite limited. Jia *et al.* (1997) described a protocol for indirect shoot organogenesis and plantlet regeneration from hypocotyls via callus phase. Yang *et al.* (2006) described an efficient plant regeneration system *in vitro* from epicotyl segments of kumquat by direct organogenesis.

In the present study, aiming on the effect of season to take shoot tip, salt levels and medium type, and plant growth regulators on initial culture of oval kumquat *in vitro* that to develop an efficient method for propagation of oval kumquat.

Material and Methods

I. Material

In the present study, sample was used taking from 3-years old oval kumquat trees growing in net-house at the Viticulture Research Station, National Chung Hsing University, Wufeng, Taichung, Taiwan.

II. Culture medium

The basal medium (Murashige and Skoog, 1962) contained 30 g/l sucrose and 8 g/l agar (Bacto-agar) for solid medium or use paper bridge for liquid medium. The medium was balanced to pH 5.7 - 5.8 with 1N NaOH or 1N HCl, dispensed in 50 ml culture tubes (10 ml medium per tube), and autoclaved at 121 ^{0}C, 1.25 kg/cm^{2} for 20 min. The plant growth regulators (BA, IBA, and kinetin) were supplemented according to every experiment as detailed below. Ten shoot tip cultures per treatment were incubated at 26 ± 2 ^{0}C under a 16 h photoperiod of cool - white light (69 μmol m^{-2}s^{-1}). The cultures were maintained for once after 21 days of incubation.

III. Methods

In the present study, active shoots were collected from different seasons. The fully expanded leaves were removed, shoot was cut with approximately 1 cm long and sterilized by 1% (w/v) sodium hypochlorite (20% (v/v) Clorox) for 5 min while shaking, then rinsed 3-4 times with sterile distilled water. Shoot tips 0.5 mm in length containing the meristem with 4 leaf primordial were excised under a microscope from sterilized shoots of kumquat and cultured upright into the culture medium. Survival percentage and fresh weight were investigated.

1. Effects of season to take shoot tip on initial culture

In this experiment, shoot tips from different seasons (spring, summer, autumn, winter) were cultured on MS solid medium adding 30g/l sucrose, 8 g/l agar, 4 treatments, 10 explants/treatment, total 40 explants. The cultures were maintained for once after 21 days of incubation, survival percentage and fresh weight were investigated.

2. Effects of salt levels and medium types on initial culture

In this experiment, spring shoot tips were cultured on 2 medium types (solid or liquid) and different salt levels included 1/4 MS, 1/2 MS, MS, 2 MS. Medium adding 30g/l sucrose, 8 g/l agar (for solid medium) or using paper bridge (for liquid medium), 8 treatments, 10 explants/treatment, total 80 explants. The cultures were maintained for once after 21 days of incubation, survival percentage and fresh weight were investigated.

3. Effects of BA on initial culture

In this experiment, MS solid medium contains 30g/l sucrose, 8 g/l agar adding different concentrations of BA at 0, 0.1, 0.5, and 1 mg/l, 4 treatments, 10 explants/treatment, total 40 explants. The cultures were maintained for once after 21 days of incubation, survival percentage and fresh weight were investigated.

4. Effects of BA and IBA on initial culture

In this experiment, MS solid medium contains 30g/l sucrose, 8 g/l agar and 0.1 mg/l BA adding different concentrations of IBA at 0, 0.01, 0.03, 0.05, and 0.1 mg/l, 5 treatments, 10 explants/treatment, total 50 explants. The cultures were maintained for once after 21 days of incubation, survival percentage and fresh weight were investigated.

5. Effects of BA, IBA and kinetin on initial culture

In this experiment, MS solid medium contains 30g/l sucrose, 8 g/l agar, 0.1 mg/l BA, and 0.01 mg/l IBA adding different concentrations of kinetin at 0, 0.05, 0.1, and 0.5 mg/l, 4 treatments, 10 explants/treatment, total 40 explants. The cultures were maintained for once after 21 days of incubation, survival percentage and fresh weight were investigated.

Results

1. Effects of season to take shoot tip on initial culture

In this experiment, shoot tips from spring, summer, autumn and winter were cultured on MS solid medium adding 30g/l sucrose, 8 g/l agar. Results shows in Table 1. In general, shoot tips survived on medium after 21 days of incubation irrespective of seasons to take shoot tip, however, highest fresh weight of explant and growth more rapid as explants were collected in spring, obtained 3 mg fresh weight.

Table 1. Effects of season to take shoot tip on initial culture of oval kumquat *in vitro*.[z]

Seasons	Survival percentage (%)	Fresh weight (mg)
Spring	100	3.0 ± 0.3[y]
Summer	100	2.5 ± 0.2
Autumn	100	2.8 ± 0.3
Winter	100	2.2 ± 0.2

z: MS solid medium contains 30 g/l sucrose and 8g/l agar, investigated on the 21st day of culture.

y: Mean ± standard error.

2. Effects of salt levels and medium types on initial culture

Survival percentage and fresh weight shows in Table 2. Survival percentage of explants were very poor on medium contains 1/4 MS salt after 21 days incubation, whereas, all explants survived at other salt levels irrespective of medium type. In general, fresh weight of explants was higher in solid medium than liquid medium. MS solid medium obtained the better result in fresh weight (3.0 mg) than other ones. Increased salt concentration to 2MS, fresh weight of explant decreased. The best result as shoot tips were cultured on MS solid medium, gave 100 % survival and 3 mg fresh weight.

Table 2. Effects of salt levels and medium types on initial culture of spring shoot tip of oval kumquat *in vitro*.

Salt levels	Solid[z]		Liquid[y]	
	Survival (%)	Fresh weight (mg)	Survival (%)	Fresh weight (mg)
1/4 MS	10	1.2 ± 0.8[x]	20	1.4 ± 0.9
1/2 MS	100	2.0 ± 0.5	100	2.0 ± 0.6
MS	100	3.0 ± 0.3	100	2.8 ± 0.3
2 MS	100	2.4 ± 0.9	100	2.2 ± 0.5

z: Solid medium contains 30 g/l sucrose and 8g/l agar.

y: Liquid medium (paper bridge) contains 30 g/l sucrose.

x: Mean ± standard error.

3. Effects of BA on initial culture

In the present experiment, shoot tips were cultured on MS medium adding 30 g/l sucrose, 8 g/l agar and different concentrations of BA at 0, 0.1, 0.5, and 1 mg/l. Results shows in Table 3. Survival was 100% as supplement of 0.1 - 0.5 mg/l BA in medium, whereas at higher BA concentrations, survival percentage and fresh weight decreased. Significant decrease in fresh weight of explants was noted when concentration of BA was 0.5-1 mg/l in medium, and increased concentration of BA to 1 mg/l, some explants showed mortality. The best result, 100 % of survival and 3.5 mg fresh weight, when shoot tips cultured on medium containing 0.1 mg/l BA.

Table 3. Effects of BA on initial culture of shoot tip of oval kumquat *in vitro*.[z]

BA (mg/l)	Survival percentage (%)	Fresh weight (mg)
0	100	2.6 ± 0.4[y]
0.1	100	3.5 ± 0.4
0.5	100	1.8 ± 0.6
1	20	1.2 ± 0.8

z: MS solid medium contains 30 g/l sucrose and 8 g/l agar, investigated on the 21st day of culture.

y: Mean ± standard error.

4. Effects of BA and IBA on initial culture

In the present experiment, shoot tips were cultured on MS medium contains 30 g/l sucrose, 8 g/l agar and 0.1 mg/l BA supplement with different concentrations of IBA at 0, 0.01, 0.03, 0.05, and 0.1 mg/l. Results shows in Table 4. Medium containing 0.01 mg/l IBA was 100% in survival, fresh weight in 4 mg was the highest. The survival percentage and fresh weight decreased while concentration of IBA increased. Some explants showed mortality at 0.05 - 0.1 mg/l IBA. The best result obtained 100 % survival and 4 mg fresh weight when shoot tips were cultured on MS solid medium adding 0.01 mg/l IBA and 0.1 mg/l BA.

Table 4. Effects of BA and IBA on initial culture of shoot tip of oval kumquat *in vitro*.[z]

IBA (mg/l)	Survival percentage (%)	Fresh weight (mg)
0	100	3.2 ± 0.4[y]
0.01	100	4.0 ± 0.3
0.03	100	2.9 ± 0.5
0.05	30	1.8 ± 0.6
0.1	20	2.0 ± 0.9

z: MS solid medium contains 30 g/l sucrose and 8 g/l agar adding 0.1 mg/l BA. Investigated on the 21st day of culture.

y: Mean ± standard error.

5. Effects of BA, IBA and kinetin on initial culture

In the present experiment, shoot tips were cultured on MS medium contains 30 g/l sucrose, 8 g/l agar, 0.1 mg/l BA, and 0.01 mg/l IBA supplement with different concentrations of kinetin at 0, 0.05, 0.1 and 0.5 mg/l. Results shows in Table 5. 100% of explants survived on medium supplement of kinetin. At 0.1 mg/l kinetin, fresh weight of explant significant increased (5.2 mg) but that decreased when concentration of kinetin increased to 0.5 mg/l. The best result obtained 5.2 mg fresh weight when shoot tips cultured on solid MS medium adding 0.1 mg/l BA, 0.01 mg/l IBA and 0.1 mg/l kinetin.

Table 5. Effects of BA, IBA and kinetin on initial culture of shoot tip of oval kumquat *in vitro*.[z]

Kinetin (mg/l)	Survival percentage (%)	Fresh weight (mg)
0	100	3.5 ± 0.4[y]
0.05	100	3.5 ± 0.5
0.1	100	5.2 ± 0.7
0.5	100	2.8 ± 0.4

z: MS solid medium contains 30 g/l sucrose, 8 g/l agar adding 0.1 mg/l BA and 0.01 mg/l IBA. Investigated on the 21st day of culture.

y: Mean ± standard error.

Discussion

Survival percentage of explants in initial culture dependent on many factors such as collecting explants, donor plants, formula of culture medium, shoot tip size, composition of medium and season to take shoot tip was also one factor of effect on shoot tip culture *in vitro*. The success rate of *in vitro* shoot tip culture can be increased by selecting the most suitable time for explant collection (Baydar *et al*., 2006).

Amin and Jaiswal (1993) reported November to January was the best season for initiation culture of jackfruit from field-grown trees. Thomas and Ravindra (1997) reported that best response of explant in mango from shoot tip was during June and August. Explants collected during other periods showed that more medium discoloration, turned brown sooner and showed no growth response. Wang (2001) indicated that bud-inducing rate of longan was significantly higher in explants taken in summer and autumn than in those taken in spring, buds sprouting in spring are not suitable as cultural material for bud induction. Since shoots begin to accumulate nutrients after sprouting in spring, they mature and more ready to grow in the summer and autumn. In the present study, shoot tip culture could be established at any time during the year, however, survival percentage, fresh weight of explant, and growth more rapid if explants were collected in spring. It is considered that kumquat in summer, flowering and fruiting season have competition of nutrients between shoot tip and other actively development organs, so decrease shoot growth and development. In other hand, environment conditions also effect on plant physiology, change nutrient contents and endogenous hormones. For these reason, growth and development of explant was higher in spring than other seasons.

Some of important factors for successful culture *in vitro* are type of medium and salt levels (Thomas and Ravindra, 1997). For salt levels, Wang (2001) reported that shoot tip culture of longan required high concentration of inorganic salt, MS media was the best, while 1/2 MS and White media were not suitable for culture. Optimal composition of macronutrients strongly depended on genotype, Skirvin *et al*. (1979) showed the most studies on *in vitro* propagation of apricots have used MS, contrary to this, MS proved to be one of the worst media in study of 'Bulida', 'Currot' and 'Helena' apricot cultivars, all explants died on this medium within six weeks (Petez-Tornero and Burgos, 2000). MS medium was almost optimum for multiplication cherry rootstock Gisela 5, but ionic composition of 2MS medium was more favourable for plant growth (Ruzic *et al*., 2000). Lux-Endrich *et al.* (2000) indicated that shoots of apple cultured on 1/4 MS and 3 MS appeared unhealthy and stressed, cultured on 1/4 MS produced red stems and curled leaves, shoots cultured on 3 MS showed chlorotic leaves and necrotic shoot tips, only those shoots cultured on 1/2MS, 1MS and 2MS looked healthy with fully expanded, green

leaves. In the present study, survival percentage was very poor when explants cultured on 1/4 MS medium, whereas, survival percentage was highest as shoot tip of oval kumquat cultured on MS medium, 100 % of survival, irrespective of medium type. Fresh weight of explants decreased when salt level increased to 2 MS, may be oval kumquat need high salt level for growth and development, but too high salt level in medium can affect explant growth in ion toxity, ionic imbalances and depressed water potential (Choi *et al*., 1998).

In medium type, liquid culture systems are better amenable to automation (Ilczuk *et al*., 2005). However, micropropagation of woody plants in liquid medium is often hampered by the phenomenon of hyperhydricity (vitrification), this often reduces multiplication rates, induce poor quality shoots and ultimately lead to tissue necrosis (Adam *et al*., 2002). In this study, using liquid medium was not different in survival rate compare to using solid medium, however fresh weight of explants was higher in solid medium than liquid medium. The best result by using MS solid medium for shoot tip culture of oval kumquat *in vitro*.

The most important factor for successful tissue culture is plant growth regulators in medium. The efficacy of different concentrations and combinations of growth regulators on *in vitro* propagation of fruit tree included citrus reports (Singh *et al.*, 1994; Paudyal and Haq, 2000). In the present study, effect of basal solid MS medium adding different concentrations of BA on initial culture of shoot tip in oval kumquat, the result was shown that survival of shoot tips were 100% at 0.1-0.5 mg/l BA, whereas higher BA concentrations, survival percentage and fresh weight of explant decreased. Significant decrease in fresh weight of explants when concentration of BA were 0.5-1 mg/l in the medium, could be too high BA concentration inhibit growth of explant in oval kumquat *in vitro*. Paudyal and Haq (2000) reported as cultured shoot tip of pummelo, survival of shoots decreased as concentration of BA increased, all shoots in hormone-free medium survival and resumed normal growth, while only 69% of shoots survived when cultured with 2.2 µM BA. Further significant decrease in survival percentage of explant was noted as concentration of BA exceeded 2.2 µM.

In some fruit tree species, shoot development was observed when shoot tips were cultured on MS medium containing with BA without other plant growth regulators (Nomura *et al*., 1998) or the combination of different plant growth regulators concentrations. Singh *et al*. (1994) reported the combination of BAP, kinetin, NAA appeared to be essential for culture in different citrus species**.** In this study, shoot tips were cultured on MS solid medium containing 0.1 mg/l BA, 0.01 mg/l IBA and 0.1 mg/l kinetin for best results in all initial culture experiments.

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長實金柑(*Fortunella margarita* Swingle)初代莖頂培養之研究

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關鍵字：物生長調節劑、金柑、組織培養

摘要：本研究目的為探討長實金柑之莖頂組織培養。由試驗觀察發現，在春季採集之新梢在顯微鏡底下切取0.5mm之莖頂，並以含有30 g/l 蔗糖與8 g/l 洋菜之MS培養基進行初代培養可得較高之存活率。將存活之培植體移植至含有30 g/l 蔗糖、8 g/l 洋菜之MS培養基並添加 0.1 mg/l BA、0.01 mg/l IBA與 0.1 mg/l kinetin繼續培養則生長表現最佳。

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